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Degree: Master of Science

Year this Degree Granted: 2001

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University of Alberta

Kinetics of Ring Opening Reactions of Dibenzothiophene by *Pseudomonas fluorescens* LP6a

By

Seena Mohammed Salem Farea ©

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of **Master of Science**
in
Chemical Engineering

Department of Chemical and Materials Engineering

Edmonton, Alberta

Fall 2001

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Kinetics of Ring Opening Reactions of Dibenzothiophene by *Pseudomonas fluorescens* LP6a submitted by Seena Farea in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering.

ABSTRACT

This project investigated a potential alternative for conventional upgrading of fuels, using a biocatalyst consisting of *Pseudomonas fluorescens* LP6a mutant #21- 41. The objectives of this study were to identify reasons for reduction in biocatalytic activity with time and to find means for rejuvenation. The system consisted of the mutant in resting state with dibenzothiophene, as the model compound, dissolved in hexadecane. The system showed a first order rate of conversion. A mathematical model was developed to predict the initial rate of conversion and was consistent with the experimental values. The freshly prepared inoculum gave a biocatalyst with high activity for a period of 96 hr. When the biocatalyst showed low activity a provision of fresh carbon source resulted in some recovery of activity, suggesting that the cells required maintenance energy. The experiments in this study showed that neither the reactants nor the end products inhibited the biocatalyst.

Acknowledgement

بسم الله الرحمن الرحيم

In the Name of God Most Gracious Most Merciful

This page was the hardest to write since I did not know whom to start thanking. Each person I met during the past years had supported me in the completion of this work. For them all I say “Thank you”.

My first acknowledgement goes to my supervisor Dr. Murray Gray, whose advice, guidance, and encouragement supported me throughout this research.

I would like to thank United Arab Emirates University for their belief in me, and their generous funding, it would not had happen without the wise management. I hope to serve United Arab Emirates in its prosperity and fulfilling the dream of the nation’s Father.

The spirit of the group work at the University of Alberta, the biological science department had supported me. For that I would like to thank Dr. Julia Foght, Beth Hearn, Kathlyn Kirkwood, Leah Kirkpatrick, Lisa Tran and not to forget the new comers to the group, In S. Kim and Dave Bressler. I would like to address a special thank to Dr. Q. Wu for his kindness and patience in guiding me.

The mental and moral support was there all the time, from my dearest sisters in Islam, Aysha, Razia, Farhana, Sylvia, Fatin, Howaida, Mona, Zahira and many others.

Through my life the greatest support was from my family, my mother Qadria, my father Mohammed, my sister Sawsan, my brother Salem, and their sweetest kids.

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List of Nomenclature and Abbreviations

2AB = 2-Amino benzoic acid.

Abiotic = Physical-chemical effects; absence of biological factors.

ATP = Adenosine triophosphate; a chemical that stores energy in phosphate bonds.

BCA = Bicinchoninic acid.

Biph. = Biphenyl.

BSA = Bovine serum albumin; a protein used as a calibration standard for protein assays.

BT = Benzothiophene.

C_{16} = Hexadecane.

C_6 = Hexane.

Catabolic repression: repression of a variety of unrelated enzymes when cell are grown in a medium containing glucose

CFU = Colony forming unit; the number of cells that grow on agar media.

CN = Cetane Number.

Cometabolism: The metabolic transformation of a substance while a second substance serves as primary energy or carbon source.

DBT = Dibenzothiophene.

DBTO = dibenzothiophene 5-oxide.

$DBTO_2$ =dibenzothiophene 5,5-dioxide.

DCM = Dichloromethane.

ddH_2O = Doubly distilled H_2O.

DMF = N,N – Dimethyl formamide.

Doubling time: the time needed for a population of bacteria to double

EtOH = Ethanol.

Exp. = Experiment.

HBP =2-hydroxybiphenyl.

HBPSi =2-(2’-hydroxyphenyl)benzene sulfinate.

HFBT = 3-hydroxy-2-formylbenzothiophene.

HMN = Heptamethylnonane, a highly branched aliphatic solvent.

HTOB = *trans*-4-[2-(3-hydroxy)-thianaphtheny]-2-oxo-3-butenoic acid.

Hybridization: The artificial construction of a double stranded nucleic acid by complementary base pairing of two single stranded nucleic acids.

Induction = The process by which an enzyme is synthesized in response to the presence of an external substance, the inducer.

Inoculum = A material used to initiate a microbial culture.

Insertion = A genetic phenomenon in which a piece of DNA is inserted into another strand of DNA, such as a gene.

Insertion Element = Specific nucleotide sequences that are involved in the transfer and integration of pieces of DNA.

IS = Internal standard.

ISRF = Internal standard reference point.

kbp = Thousand base pairs; a one-thousand base fragment of nucleic acid.

M = Molarity.

Mutant = A strain differing from its parent because of mutation.

Mutation = An inherited change in the phenotype of an organism.

Mwt. = Molecular Weight.

N = Normality.

NAD and NADH = Nicotinamide-adenine dinucleotide, oxidize and reduced forms.

NADP and NADPH = Nicotinamide-adenine dinucleotide phosphate, oxidize and reduced forms.

NAPL = non-aqueous phase liquid

OD_{600} = Optical density at absorbance 600 nm

ORFs = Open reading frames.

PAC = Polynuclear aromatic compounds.

PCA = Plate Count Agar.

Phenotype: the observable characteristics of an organism.

Rt. = G.C. retention time.

SA = Salicylic acid.

Stationary Phase = The period during the growth cycle of a population in which growth ceases.

t = Time.

Tn = Transposon.

TSB = Tryptic Soy Broth; a medium for growth.

List of Symbols

AU = Absorbance unit

c = Concentration of solute

Co = Organic concentration of DBT in bulk hexadecane phase (µmole.mL^{-1})

Coi = Organic concentration of DBT at equilibrium with the aqueous phase (µmole.mL^{-1})

Cw = The aqueous concentration of DBT (µmole.mL^{-1})

Cwi = Aqueous concentration of DBT at equilibrium with the hexadecane phase (µmole.mL^{-1})

Flux = The movement of DBT between the two phases (µmole.m^{-2}.hr^{-1})

g = Gravity 9.8 (m^{2}.s^{-1})

k = Overall mass transfer coefficient in the aqueous phase (m.hr^{-1})

k$^{/}$ = Overall mass transfer coefficient in the hexadecane phase (m.hr^{-1})

k$_{P}$ = Dimensionless partition coefficient (mole DBT.L^{-1} of hexadecane) / (mole DBT.L^{-1} of water)

L = Length of light path

ε = Molar extinction coefficient of solute

1.0 INTRODUCTION

The extracted bitumen from Alberta is extensively treated to form a synthetic crude oil for further trading in the oil market. These conventional treatments are referred to as bitumen upgrading. The middle distillates resulting from these treatments have high concentrations of naphthenic and aromatic type structures that cause poor properties in diesel fuel. The conventional upgrading techniques require high temperature and high pressure processes to cleave and hydrogenate fused-ring polycyclic aromatic compounds (PAC). These severe conditions still do not provide high selectivity reactions for the desired products.

A potential alternative for conventional upgrading is to use a biocatalytic system to selectively open aromatic structures to form oxygenated species, followed by chemical hydrogenation processes to form alkylaromatic structures. The alkylaromatic compounds provide high quality diesel fuel. The technique was developed using *Pseudomonas fluorescens* strain LP6a that degrades a wide range of PAC such as naphthalene, phenanthrene, dibenzothiophene and alkylnaphthalenes. A transposon mutant of LP6a, mutant #21-41, had its degradation abilities modified to oxygenate PAC to an open ring structure without excessive degradation.

In general, conversion is the critical step of any biocatalytic process, depending on specificity, selectivity, activity and stability of the biocatalyst. Mutant #21-41 possesses these characteristics required for oil upgrading to diesel fuel. The specificity is linked to the microbial capacity to open the ring structure without changing the carbon

content and thus its fuel value. The conversion selectivity is due to the ability of the mutant to convert aromatic structures in middle distillate, without transforming the aliphatic chain, which are important hydrocarbons making up diesel fuel. This biocatalytic tuning of middle distillate or pre-produced diesel fuel has the potential to perform better than the conventional methods because of the moderate temperature and pressure used in the biotechnology-based approach, which will result in significantly lower capital and operating expenses.

In addition, mutant #21-41 retains cleavage activity after being grown on inexpensive media such as molasses. The biocatalytic stability of the mutant phenotype is high, and extraordinary measures are required to generate spontaneous deletion mutants or cured derivatives. Meanwhile, if the biocatalytic stability is considered as the sustained ability to function within a biphasic system, then mutant #21-41 is able to transform PAC existing in a water-immiscible aliphatic carrier system.

Previous studies optimized the biocatalyst conditions, and identified the major open ring metabolites from different PACs (Wu & Foght, 1997). Some studies with *Rhodococcus erythropolis* KA2-5-1 showed declining biocatalytic activity with repeated biocatalyst usage (Naito et al., 2001), and similar results were seen when some PAC were solubilized using different organic carriers. However, those studies did not specify if the declining activity was due to inhibition by open ring metabolites, or the exhaustion of the biocatalyst. This work investigated some of the factors affecting the biocatalyst activity and studied the means by which the biocatalyst can be recycled.

Dibenzothiophene was selected for use as the model compound, because of its low volatility. Moreover, the metabolites of interest from DBT were water soluble, with an absorbance maximum at 475 nm in phosphate buffer at pH 7.3 (Kodama, et. al., 1973; Hou & Laskin, 1975), thus they were easily detected by the spectrophotometer. DBT was solubilized in hexadecane as an organic carrier. Hexadecane was chosen because of its low volatility, low toxicity and the mutant's inability to degrade it.

The first objective of this research was to determine if DBT metabolites were inhibiting mutant 21-41 from further conversion of DBT. The second objective was to find suitable means to recycle the biocatalyst and study possibilities of rejuvenation of enzyme activity. There were several factors to be considered, including exhaustion of the cell energy, and the removal of inhibitory metabolites. The activity of the biocatalyst was determined by monitoring the rate of conversion of DBT. Since the model compound DBT had an open ring product with maximum absorbance at 475 nm, whenever possible the intensity of the colored metabolite was used as a measure of biocatalyst activity, and complemented by a direct gas chromatography measurement of the remaining (unconverted) DBT. The gas chromatography had a flame ionization detector (GC-FID)

2.0 LITERATURE REVIEW

2.1 Bitumen Processing in Alberta

Tar sands, or bituminous sands, are deposits of loose sand or partially consolidated sandstone that are saturated with highly viscous bitumen. Alberta bitumen makes up approximately three-quarters of the total world endowment. The extracted bitumen is extensively treated to form a synthetic crude oil for further trading in the oil market. These treatments are referred to collectively as bitumen upgrading.

In the case of Alberta bitumen, several studies revealed that the chemical distribution of the hydrocarbons were the reason for its poor fuel characteristics. For example, about 25-35% of the total carbon in Alberta bitumen is dominated by aromatic structures, and the concentration of *n*-alkanes is lower than the amount in conventional petroleum. Approximately 11-14% of the total carbon exists in long paraffinic side chains on the cyclic and aromatic compounds, with these paraffinic bridges linking the different aromatic and naphthenic rings (Gray, 1990). When such distributions of chemical structures are cracked, they produce middle distillates with high concentrations of naphthenic and aromatic type structures that have poor properties for diesel fuel. These product streams require further treatment in the oil upgrading and refining processes.

The current upgrading technology involves several possible steps, which can be combined into two major categories: primary upgrading and secondary upgrading. The upgraded products must meet several downstream properties, such as providing naphtha

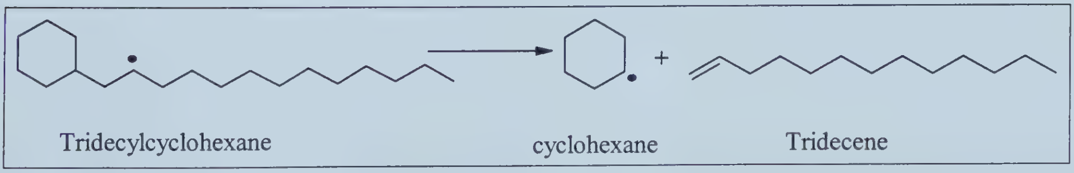
cut that has low nitrogen concentration, and a middle distillate cut suitable for diesel blendstock (Gray, 1990).

2.1.1 Primary Upgrading

This first stage of the upgrading process produces the distillate products by means of thermal reactions. Thus, primary upgrading takes place under severe conditions of high temperatures above 420°C, which aims to break the high molecular weight compounds into lighter ones by breaking the alkyl C-C and C-S bonds. There is no commercial benefit in using a catalyst at that stage, since any catalyst that may facilitate the reaction is rapidly inactivated by heteroatom adsorption on the active sites (Mills, 1950).

It is believed that in the thermal cracking process, free-radical chain reactions break the alkyl C-C and C-S bonds. The paraffinic structures are easier to crack than the cyclic olefins or aromatic structures. For the paraffinic side chains of the naphthenic rings, one of the favorable positions for breakage is the adjacent point to the cyclic structure, as shown below in Figure 2-1 (Gray, 1994).

Figure 2-1: *β*-scission reaction of cycloalkanes (Gray, 1994)



Aromatic hydrocarbons with short alkyl groups (<C3) are not very reactive (Gary & Handwerk, 1984). However, the predominant cracking reaction for alkylaromatics with long side alkyl chains will generate structures such as alkylaromatics with short chains, alkanes, olefins, and free radicals. The generated free radicals in later stages promote condensation reactions, forming fused-rings and coke. As an example, Savage and Klein (1987) showed that the pyrolysis of *n*-pentadecylbenzene, as illustrated in Figure 2-2, gave toluene and styrene, and formed a low yield of phenyl alkanes and phenyl olefins with chains of 2-12 carbons. Moreover the formed alkylaromatic radicals tend to participate in condensation reactions that lead to coke formation (Savage & Klein, 1987). These condensation processes are emphasized with larger aromatic structures at lower temperatures, where they undergo addition reactions as shown in Figure 2-3, and the bridged structures add together to finally form coke (Gray, 1994).

As illustrated the thermal cracking reactions tend to leave many of the aromatic groups intact as well as the naphthenics and naphthenoaromatics, plus many of the heteroatoms are present in less reactive heterocyclic compounds. Therefore, secondary upgrading is needed to modify these compounds to meet the downstream product re.

Figure 2-2: Pyrolysis of *n*-pentadecylbenzene (Savage & Klein, 1987)

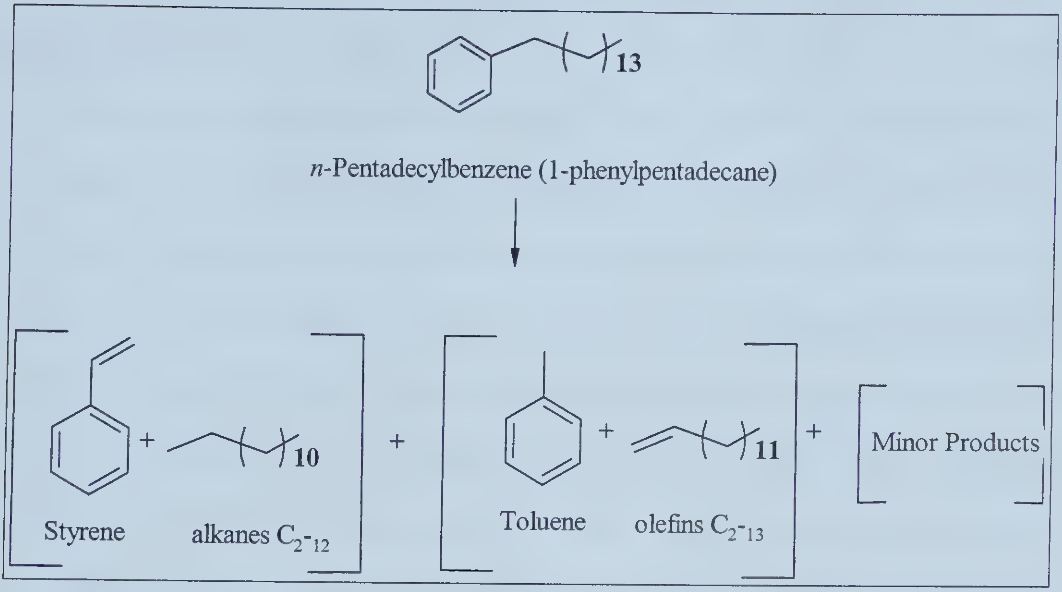
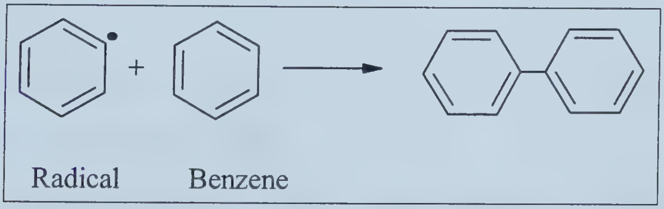

13
n-Pentadecylbenzene (1-phenylpentadecane)
+
10
Styrene
alkanes C2-12
+
+
11
Toluene
olefins C2-13
+
Minor Products

Figure 2-3: Condensation reactions of alkylaromatic radicals (Gray, 1994)


+
Radical
Benzene

2.1.2 Secondary Upgrading

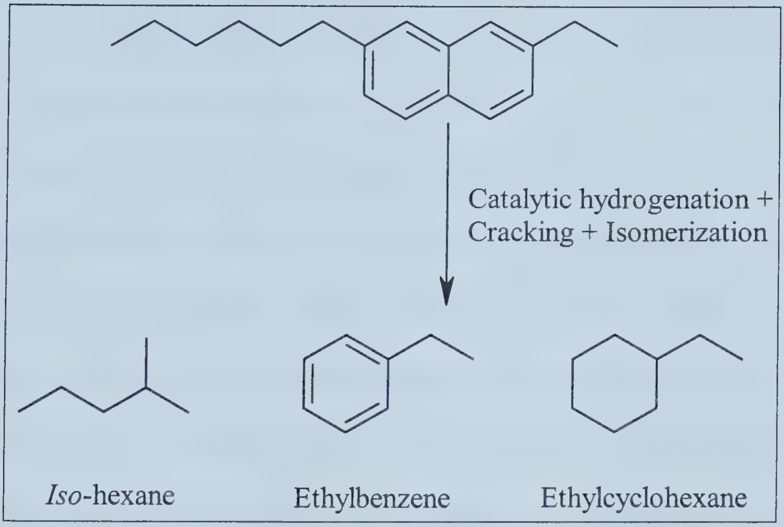
This stage of oil upgrading is designed to selectively modify the distillate products of the primary upgrading units, by removing the heteroatoms, and converting the remaining multi-ring aromatic compounds to more desirable compound structures. The whole process takes place under mild hydrotreating conditions of temperature lower than 400°C with the aid of a metal catalyst (Gray, 1994). The catalysts most generally used are cobalt and molybdenum oxides on alumina. Usually the hydrotreatment process occurs in a fixed bed reactor with the liquid stream and the hydrogen gas flowing downward co-currently, and the reaction takes place at the catalyst surface. Figure 2-4 presents a typical hydrocracking reaction where the complex alkyl aromatic compounds are further cracked to short highly branched chains, alkylaromatics and alkyl-cyclic structures with short side chains (Gary & Handwerk, 1984). These products are useful for gasoline fuel, but one of Alberta's upgrading units objectives is to produce high quality diesel fuel.

From the pervious presentation, both stages of oil upgrading had major drawbacks from the perspective of product quality:

- The large clusters of polynuclear aromatic compounds (PACs), either generated in situ or preexisting, are left without major alteration.
- The reaction kinetics (such as free radical cracking) tend to generate undesired by-products.
- The reaction conditions of high temperature, high pressure, and expensive catalyst still do not give control on reaction selectivity.

Despite these drawbacks in the conventional upgrading technology, the transportation fuel market still demands high quality fuels, such as gasoline and diesel.

Figure 2-4: Typical hydrocracking (Gary & Handwerk, 1984)



2.1.3 Diesel Quality and Cetane Number

An ideal diesel fuel has several characteristics related to its readiness to ignite and burn quietly, cleanly and economically during the compression stroke of an engine. One of the measurements that help in identifying these characteristics in diesel fuel is called the Cetane Number (CN). The CN indicates the readiness of the fuel to ignite spontaneously under the temperature and pressure conditions of the combustion chamber in the diesel engine. This is determined for a fuel by comparing its ignition delay, under standard conditions, with blends of two reference fuels, cetane (hexadecane) and heptamethylnonane, having by definition, CN of 100 and 15, respectively. For example,

the middle distillate cut from the Alberta bitumen has a low cetane number, and the specifications for diesel fuel usually require a minimum CN value of 40. Thus, to have a high cetane number, the diesel fuel must have a significant amount of saturated (paraffins and naphthenes), and alkylated aromatic or cyclic compounds with long alkyl chains (Song et al., 2000).

Although paraffinic hydrocarbons are attractive for diesel fuel, they may pose problems in meeting low-temperature specifications for pour point. However, oil companies generally vary the mix of hydrocarbons in the diesel fuels to satisfy the consumer demands. For example, they may include more compounds of low boiling range for the cold climate markets, and vice versa for hot weather markets. These mixes of hydrocarbons occur due to blending of different refinery streams, which vary in the content of the different chemical hydrocarbon structures. The hydrocarbons found in diesel fuel are paraffins, naphthenes, and aromatics; their proportions determine the diesel efficiency. The major paraffins in diesel are in the range of C_{10}-C_{20}, with minor amounts of the lighter ($<C_9^-$) and the heavier ($>C_{20}^+$). The fraction of naphthenic components present (e.g. alkylated cycloalkanes) depends on the nature of the crude, and the processing parameters as shown in section 2.1.2, Figure 2-4. The aromatic compounds include alkylated benzenes, naphthalene, phenanthrene and pyrenes, with an abundance of the diaromatic hydrocarbons (Song et al., 2000). However, not all these components are available together in high concentration. The availability of each chemical structure depends on the nature of the crude and the upgrading and refining processes. For example, a paraffinic crude processed to a straight run distillate gives a high CN diesel fuel, but such combinations of crude oil and controlled processes are

limited. Alternatively, the modifications to the available feedstock can also increase the CN. For instance, a stream rich in cycloalkanes can be treated to crack the rings and increase the CN. Although aromatic saturation can increase the CN, yet some studies have shown that complete hydrogenation of the aromatics is not possible under conventional hydrotreating conditions described in section 2.1.2. Therefore, for complete hydrogenation a noble metal catalyst has to be used under low temperatures, but these catalysts are sensitive to sulfur deactivation.

The addition of chemicals downstream can also improve the diesel CN. Additives used as cetane improvers are reactive compounds that can generate free radicals and favor auto-ignition in the engine. With these additives it is possible to increase the CN by 3 to 6 numbers, but unfortunately it is the fuels that have the lowest CN that tend to respond least to cetane improvers. Moreover, cetane improvers generally contain nitrogen compounds that may cause additional environmental problems (Song et al., 2000).

As mentioned in section 2.1, middle distillates from Alberta oilsand bitumen have a high aromatic content which produces a low CN. The same problem with gas oils from catalytic and thermal cracking units is that they tend to have lower ignition quality (Owen & Coley, 1990). With the difficulties of conventional oil upgrading processes in improving the CN and their environmental limitations, new options must be considered: either additional improvement on the current upgrading processing units, or introducing a novel upgrading technology. By proceeding with the second option, this thesis will introduce the utilization of a microorganism that selectively upgrades the PAC, such as naphthalene, phenanthrene, biphenyl, and dibenzothiophene, with the ultimate aim of producing diesel fuel with a high CN from aromatic middle distillates.

2.2 Biocatalytic Biotransformation

Several microorganisms are known for generating enzymes to either degrade or synthesize a great variety of chemical compounds. Therefore, the ultimate goal of industrial biotechnology has been to utilize such capabilities in microorganisms to generate useful products by successfully adapting the scientific ideas that were developed in the laboratory and turn them into economic realities (Jackson, 1991). A biocatalyst refers to a sufficient biomass of a microorganism with a particular active enzyme. The active enzyme provides a reaction surface for the substrate. Therefore, the biocatalyst may include a viable microorganism with the active enzyme or the purified enzyme.

2.2.1 Forms of Biocatalyst

There are several factors that contribute to finalizing the optimum design for a biocatalyst in batch processing. The choice of whether to use living cells or purified enzymes is decided based on availability, expense and the reaction environment. Examples of such factors are: (1) whether the membrane of the cells prevents the transfer of either the substrate or the product, and (2) whether the useful enzymes are functional only within the intact living cell. Table 2-1 summarizes the different advantages and disadvantages of both strategies (Roberts et. al, 1995).

Table 2-1: Advantages and disadvantages of whole-cell systems and purified enzyme systems (Roberts et. al, 1995).

Biotransformation System	Advantages	Disadvantages
Whole Cells	-Inexpensive -Enzyme co-factors present	-Require large vessels -Messy work-up -Side reactions can interfere or dominate -Substrate and/or product, and/or co-solvent may disrupt (membrane-bound) enzymes
Isolated enzymes	-Simple apparatus -Simple work-up	-Expensive -Addition of enzyme co-factor required -Enzyme co-factor recycling necessary

For the current work, the whole cell biocatalytic system will be introduced in the following sections. There are different types of preparations for the whole-cell culture biocatalyst including: (i) growing culture, (ii) resting culture and (iii) immobilized culture. Each one of these forms has its strength and weaknesses.

2.2.1.1 Growing Culture

In a growing culture biocatalytic system, the substrate is added either at the time of inoculation or at later stages of growth. The two main drawbacks of this type of biocatalyst are the risk of contamination with foreign microorganisms, and the difficulty in isolation and purification of the required product (Rosazza, 1982; Konishi et al., 1997).

2.2.1.2 Resting Culture

Resting cells are live, non-growing cells that retain many of their enzyme activities. To prepare them, they are first grown to optimum cell density. Then they are removed from their spent medium, and resuspended in an incomplete medium (e.g. one

without a nitrogen source) to avoid contamination by other microorganisms, and to maintain the cell density relatively constant. Then the substrate is added and the cells are incubated in an appropriate physical environment. High cell densities can be used to speed up the process (Rosazza, 1982), which makes some biotransformation processes more commercially viable (Reichmuth et al., 2000). The advantages of this two steps process are as follows:

1. *Each step can be optimized (e.g. cell density, substrate concentration, etc.)*
2. *The possibility of medium and product interaction is eliminated.*
3. *The separation of the required product is easier.*

2.2.1.3 Immobilized Culture

Immobilized cultures are mainly based on growing a pure culture of microorganism in an appropriate medium, after which the cells are harvested, washed and immobilized. The immobilizing surface or gel can be a polymer matrix, a solidified form of cellulose, or others. This form of biocatalyst is attractive because it shows higher operation stability and is easily removed from the reaction vessel where it can subsequently reused. However, upon comparison with an equivalent volume of growing cells, the catalytic activity of the immobilized culture is lower. There are several reasons for this reduced activity, which includes cell damage during the immobilization process, and rate limits on the diffusion of the substrate and /or the product from the medium to the cells (Roberts et al., 1995). The selection between these different biocatalyst preparations depends on the process objective and the economical availability.

Having described the biocatalyst as a useful biomass of microorganisms, it is necessary to understand some of the elements in these microorganisms that are involved in biotransformation. One of these elements is the genetic regulation and control of biotransformation.

2.2.2 Plasmids and Transposable Elements

A typical bacterial strain will have a single chromosome that encodes all (or most) of the genetic information of the cell, along with other non-chromosome genetic elements. The key properties of these genetic elements are their genes, and their ability to self-replicate independently from the chromosome. Two types of genetic elements are plasmids and transposable elements (Madigan et al., 1997).

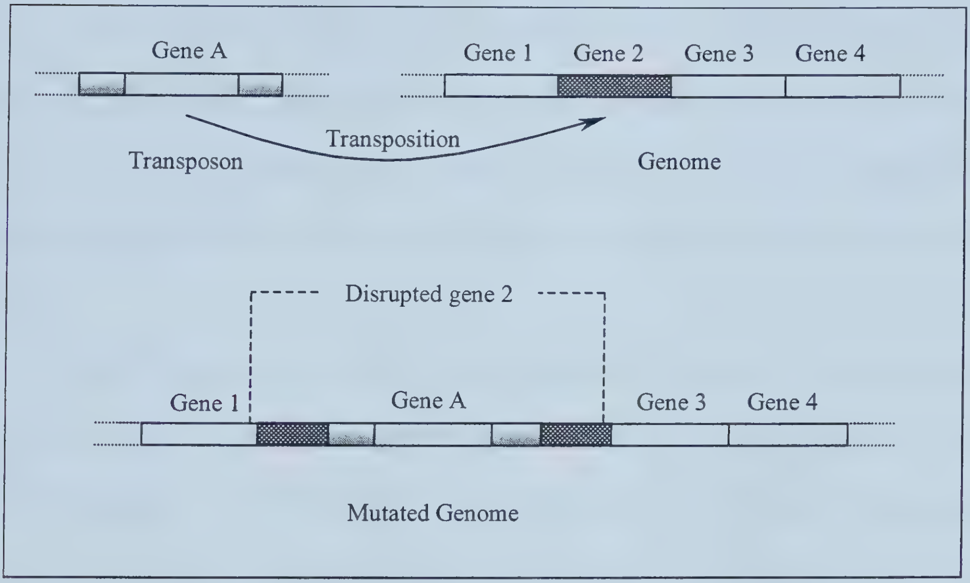
Plasmids vary in size from 1 to more than 200 thousand base pairs (kbp) of DNA, whereas bacterial chromosomes have a typical size of 1,000 to 5,000 kbp. There may be little or no nucleotide sequence homology between the plasmid DNA and the chromosomal DNA. Plasmid DNA rarely encodes functions that are critical for cell growth in the laboratory, however, many plasmids are important to survival of the bacterial in its natural environment (Neidhardt et al., 1990). Under controlled lab conditions that prevent the loss of the plasmids, many bacterial strains show special characteristics due to genes carried on the plasmids. These characteristics can be of medical, agricultural, industrial and environmental importance (Smith & Sockett, 1990). For some research purposes, plasmids are deliberately removed from the host bacterial strain to generate a cured strain. Such a curing process produces a reference strain that lack the phenotype associated with the removed plasmid, thus facilitating studing of its

role. Plasmids are eliminated from the host cell by various treatments, which inhibit the plasmid replication without parallel inhibition of the chromosome replication, and as a result of cell division the plasmid is diluted out (Madigan et al., 1997). Besides curing to remove plasmids, mutating the plasmids changes the base sequence of the DNA, which alters the cell phenotype rather than eliminating it.

Mutation can occur either spontaneously or due to chemical treatment. Some types of mutation delete segments of the DNA, while other forms add additional copies of the DNA. A change in the order of genes in DNA can cause mutation, where the gene order is reversed or interrupted by a genetic element. The latter mutation process is referred to as transposition or translocation. In translocation, the linear continuity of the genome is interrupted by an insertion of a genetic element. This interruption inactivates the genes in which the insertion occurred. The transposon (*Tn*) is one of the genetic elements involved in translocation. Some transposons have the property of replicative translocation, whereby a copy appears at a new location on the genome while the original copy remains intact. This process is illustrated in Figure 2-5, where *Tn* is inserted in gene 2 causing the interruption of its linear continuity and thus ceased its activity (Neidhardt et al., 1990). The most convenient transposon is one that contains an antibiotic resistance gene as a marker. Therefore, *Tn5* is a good candidate transposon, which carries genes for kanamycin and belomycin resistance (Ahmed & Podemski, 1998). Because *Tn5* insertion confers an antibiotic resistance property on the mutant, it assists in selecting the mutated strain from non-mutated ones. To maintain the mutation and the transposon the biocatalytic system should contain a concentration of the antibiotic drug. Apart from altering the genetic structures to form new mutants with varying phenotypes, it is

necessary for these mutants to control their enzymatic activity. Even the non-mutated strains must have a means by which they control their enzymatic activity. The controlling factors can be internal within the cells or from the environment.

Figure 2-5: Transposon mutation (Madigan et al., 1997)


Gene A
Gene 1
Gene 2
Gene 3
Gene 4
Transposition
Transposon
Genome
Disrupted gene 2
Gene 1
Gene A
Gene 3
Gene 4
Mutated Genome

2.2.3 Factors Controlling the Enzymatic Activity

Microorganisms have genetic information to encode numerous kinds of proteins, only some of which are actually present in the cell under any particular condition. Microorganisms tend to regulate their biochemical reactions in response to the external environment and/or as part of their development process. There are two main methods of regulation; one controls the *activity* of the preexisting enzyme, while the other controls the amount of enzyme *synthesis* (Madigan et al., 1997). The former will be discussed under the term enzyme inhibition and the latter under enzyme induction and repression.

2.2.3.1 Enzyme Inhibition

The regulation of enzyme activity is referred to as enzyme inhibition or inactivation. These regulatory mechanisms can be classified into different categories, depending on the context. One categorization was introduced in Madigan et al. (1997) as product inhibition and feedback inhibition. Product inhibition occurs primarily when the product of the enzymatic reaction is not used in subsequent reactions; therefore, the product accumulates and drives the enzymatic reaction in the reverse direction. On the other hand, feedback inhibition happens when one or more of the products further down the reaction pathway inactivates the first enzyme in the reaction pathway (Madigan et al., 1997).

Another categorization is the classification into two types; irreversible and reversible inhibition. The former entails the destruction or the modification of one or several functional groups of the enzyme. Meanwhile reversible inhibition is characterized by a rapid dissociation of the enzyme-inhibitor complex. This inhibition may be either competitive or noncompetitive. In competitive inhibition, the percentage inhibition of the enzyme is controlled by the concentration ratio between the competitive inhibitor and the substrate. Thus, increasing the substrate concentration prevents the competitive inhibition because all the active sites are filled by the substrate. On the other hand, noncompetitive inhibition is due to the formation of inactive complexes, where the inhibitor binds at a locus on the free enzyme on an existing enzyme/substrate complex (Lehninger, 1970). In contrast, irreversible inhibition or inactivation can be due to chemical changes in the enzyme that alters its activity permanently. For example, enzymes that utilize molecular oxygen as a substrate and enzymes that use or produce hydrogen peroxide can be

inactivateed by oxidation of the enzyme. This oxidative inactivation process may trigger the intracellular turnover of the potential enzymes (Gray, 1989). All of these mechanisms of inhibition or inactivation change the amount of enzyme activity from the initial level, with adding or removing enzyme molecules.

2.2.3.2 Induction and Repression

The other technique for cells to regulate enzymes is to regulate the synthesis of the enzyme by regulating the gene expression at the level of transcription. This type of regulation is strongly controlled by the existence or absence of certain molecules in the environment where the microorganism is growing (Madigan et al., 1997). When these molecules are present in the cell environment, they may induce the synthesis of certain enzymes. For example, *Pseudomonas saccharophila* P15 was reported to degrade phenanthrene via salicylate as an intermediate. When the strain was preincubated with salicylate, it stimulated both the activity of initial PAC degrading enzyme and the initial rates of phenanthrene removal, which suggested that salicylate was an inducer. Salicylate also greatly enhanced the initial rates of removal of other aromatic substrates, which the strain did not use for growth (Chen & Aitken, 1999).

On the other hand, the presence of other molecules may repress the enzyme synthesis. One such type is referred to as catabolite repression, which occurs when the microorganism is offered an energy source and at the same time a more readily utilizable energy source is present, such as glucose. For example, a *Pseudomonas alcaligenes* isolate DBT2 was able to oxidize dibenzothiophene (DBT) upon induction with naphthalene or salicylate. However, the presence of succinate in the growth medium

inhibited the degrading ability and increased the lag period before the cells began degrading DBT to its colored metabolites. The lag period was directly proportional to the initial concentration of succinate (Monticello et al., 1985). There was significant delay in the formation of the colored metabolites from DBT degraded by *Rhizobium meliloti* Orange 1 when the culture medium contained additional succinate or acetate as carbon source (Frassinetti et al., 1998). Therefore, in selecting a biotransformation system, knowledge of inhibition, inactivation, induction and repression are important to prepare and sustain active biocatalyst.

2.2.4 Energy and Enzyme Requirements for Biotransformation

Glucose and other nutrients are reactants in the microorganism's fueling reactions. In general fueling reaction pathways must supply adenosine triphosphate (ATP) and nicotinamide-adenine dinucleotide (NAD/NADH) and NAD-phosphate (NADP/NADPH). ATP is the means by which the cell temporarily stores the chemical energy derived from nutrients for subsequent use for varying biological tasks. Meanwhile, the coenzymes NAD/NADH & NADP/NADPH are compounds that serve as reservoirs and carriers of hydrogen atoms and oxidizing or reducing power.

Glucose in many *Pseudomonas* species is consumed to generate energy through the Entner-Doudoroff pathway. In this pathway, glucose is converted to pyruvate, which enters the citric acid cycle to generate ATP, NADH, and CO_2 (Madigan et al., 1997). Some microbial enzymes are regulated primarily by the concentration of ATP, and when the cell is deprived of a substrate for long periods, the concentration of ATP decreases slowly and the cell dies. As a result, any decrease in the cell energy pool stimulates ATP

formation and inhibits its utilization. Conversely an increase in the energy pool has the reverse effect (Neidhardt et al., 1990).

Coenzymes make it possible for chemically dissimilar molecules to be coupled as initial electron donors and ultimately electron acceptors with the coenzymes acting as intermediary or carrier. The NAD/NADH functions primarily in oxidation-reduction reactions, while NADP/NADPH functions in biosynthetic reactions (Neidhardt et al., 1990). The oxidation-reduction reactions may proceed in three stages: (1) removal of electrons from the initial donor, (2) transfer of electrons through a series of carriers, and (3) the addition of electrons to the terminal acceptor. A different enzyme catalyzes each step, and each enzyme binds to its substrate and to its specific coenzyme. Oxidation-reduction reactions may involve the transfer of hydrogen atoms. NAD^+ is a hydrogen atom carrier and always transfers two hydrogen atoms to the next carrier in the reaction chain. Such hydrogen atom transfer is called dehydrogenation (Madigan et al., 1997). When the cells are deprived of cofactors, the addition of glucose serves as a cosubstrate in the cofactor regeneration process (Bianchi et al., 1997).

2.3 Biodegradation and Transformation of Aromatic Compounds

Biodegradation involves multi-stage reaction pathways, which successively convert biopolymers or macromolecules of complex compounds to provide nutrients. On the other hand, biotransformation is a more selective reaction where a defined compound is converted into a defined final product (Rehm & Reed, 1984). Both biotransformation

and biodegradation can be carried out either with pure or mixed cultures of microorganisms, or with purified enzymes.

Several genera of bacteria are well recognized for their ability to biotransform or biodegrade PACs. Examples of such genera are *Pseudomonas*, *Alcaligenes*, *Bacillus* (Yamada et al., 1968) and *Rhizobium* (Frassinetti et al., 1998). Some bacterial strains utilize PACs for growth as carbon and energy sources, while they transforms other PACs without using them as carbon or energy sources. The former are called metabolizing reactions and the latter are called cometabolizing reactions. Some microorganisms transform heterocyclic compounds merely to remove a particular portion of the molecule without further degradation of the parent compound, such as the utilization of DBT by *Rhodococcus rhodochrous* IGTS8 as a source of sulfur (Setti et al., 1999).

Biotransformations may offer several economic advantages over chemical processing. For example, biotransformation takes place under mild conditions of temperature and pressure, which saves both operation energy and capital investment in the long run. The substrates are attacked in the required sites to produce a specific product, and several reactions can be part of the transformation or even programmed to proceed in a controlled sequence. Therefore, there has been a growing interest in replacing the current chemical oxidation process for PACs by mild biotransformations or enzyme-mimicking processes (Rosazza, 1982).

2.3.1 Mechanism for Bacterial Attack on Hydrocarbon Compounds

Several hydrocarbon substrates are present at high concentration in the environment, but they have low solubility in water. Even with a highly active enzyme, to

achieve biotransformation the active enzyme and the substrates have to be in direct contact. Therefore, microorganisms use different physiological adaptations to grow on these substrates. These mechanisms vary depending on the microorganism species and strain, the culture growth conditions, the culture age (Rosenberg and Rosenberg, 1985), and the physical state of the hydrocarbon (liquid or solid) (Thomas et al., 1986).

Some of these mechanisms involve (1) a physical contact with the hydrophobic interface, (2) the utilization of the solubilized hydrocarbon in the aqueous phase, or (3) the production of extracellular surface-active materials, which increase the emulsification of the hydrocarbon (Neufeld et al., 1980). For microorganisms using solid hydrocarbons, growth is more difficult because of the inability of a solid material to penetrate the cell membrane (Johnson, 1964) and the large amount of energy needed to disperse the solid (Thomas et al., 1986). In the case of microorganisms utilizing of solubilized hydrocarbon, Wodzinski & Johnson (1968) found that the generation times of some bacterial isolates growing on aromatic hydrocarbon were related to the solubility of the aromatic hydrocarbons. For example, the bacterial growth on naphthalene, phenanthrene and anthracene was fastest compared to naphthacene, with the former compounds having higher water solubilities (Wodzinski & Johnson, 1968, Wodzinski & Bartolini, 1972, Thomas et al., 1986). However, the concentration of the solubilized portion of hydrophobic substrates may become a growth-limiting factor. For example, fewer cells were grown in a medium containing hexane, while many more were grown if the hexane concentration in the medium was kept below the saturation level. The reason for this limitation was the partial destruction or disorganization of the membrane phospholipid of some cells (Janson, 1968).

Two immiscible liquid phases provide a distinct interfacial boundary to which cells can attach if they have hydrophobic surfaces. The extent of accumulation of cells on the interface depends on the degree of interfacial tension (Mudd & Mudd, 1924). Some strains may retain hydrophobic surfaces, for example, *Flexibacter aurantiacus* CW- 7. This strain was always oriented with its end directed to the hydrocarbon-water interface, because the major cell surface was hydrophilic with the hydrophobic portion directed toward the interface (Marshall & Cruickshank, 1973). Alternatively, with *Acinetobacter calcoaceticus* RAG-1, thin fimbriae were a major factor in its adherence to hexadecane (Rosenberg et al., 1982). Adherence to hydrocarbons is not an exclusive property of hydrocarbon degrading microorganisms (Rosenberg et al., 1980a), but it does ensure initial contact for such strains.

Another mechanism to increase the availability of hydrocarbon to microorganisms is the addition of biological or chemical surfactant. The surfactants will increase the solubility of hydrocarbons by forming micelles. Several strains are known to produce biosurfactant compounds, which have potential application in enhanced oil recovery (Finnerty et al., 1983). However, the addition of chemical compounds often shows contradictory results. Some studies showed positive results, while others found that addition of surfactant inhibited the biodegradation. One such study investigated two bacteria that degraded naphthalene and phenanthrene, namely *Pseudomonas* sp. strain 9816/11 and *Sphingomonas yanoikuyae* B8/36. Under the same experimental conditions, the addition of nonionic surfactant Triton X-100 had two different effects; it increased the conversion of the PACs for the strain 9816, and inhibited the biotransformation of both PACs in strain B8/36 (Allen et al., 1999). This apparent contradiction could be solved if

more studies focused on the role of surfactants in controlling the hydrocarbon diffusion to the cells, and whether surfactants alter the cell characteristics both internally and externally (Stelmack et al., 1999).

All of the above mechanisms may interact together, forming a more complex system that requires fundamental research to understand and identify the limiting step(s). These studies will improve the practical applications of microorganism with capabilities to utilize PACs. Examples of the practical application are the use of biocatalyst to biotransform PACs to a more useful form and the elimination of PACs in the environment by biodegradation.

2.3.2 Selective Biotransformation Pathways of Aromatic Compounds

The microbial degradation of aromatic compounds through ring cleavage is mainly an aerobic process where oxidation is the initial step. In these oxidation reactions, one or two oxygen atoms are incorporated into the aromatic structure by the microorganism. At first, these reactions were generally thought to be limited to primitive organisms such as soil bacteria. However, later this type of reaction was confirmed to exist in eucaryotic organisms extending from fungi to mammals (Ensley et al., 1982; Wackett, 1997). Some of the early studies investigated the first steps of benzene oxidation to determine whether the products coincide with the degradation of other aromatic compounds. Marr and Stone (1961) demonstrated that benzene was metabolized through catechol to *cis,cis*-muconic acid by a *Mycobacterium rhodochrous* strain and a *Pseudomonas aeruginosa* strain as shown in Figure 2-6. Later, a mutant strain of

Pseudomonas putida produced *cis*-benzene glycol, which in other microorganisms was transformed into catechol as shown in Figure 2-7 (Rosazza, 1982).

Figure 2-6: Proposed pathway of benzene oxidation (Marr & Stone, 1961)

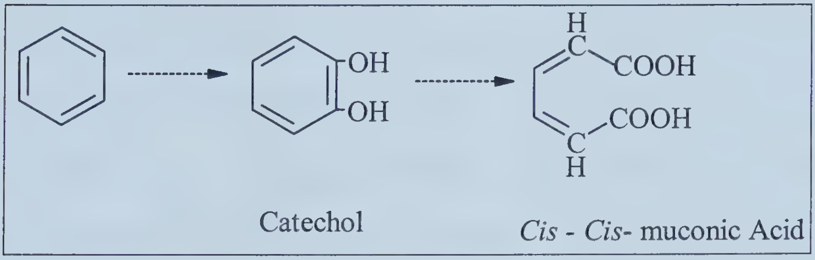
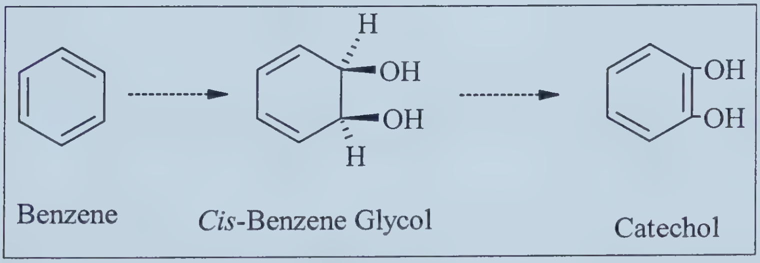

OH
OH
H
C
COOH
COOH
C
H
Catechol
Cis - Cis- muconic Acid

Figure 2-7: Microbial transformation of benzene to catechol (Rosazza, 1982)


H
OH
OH
H
OH
OH
Benzene
Cis-Benzene Glycol
Catechol

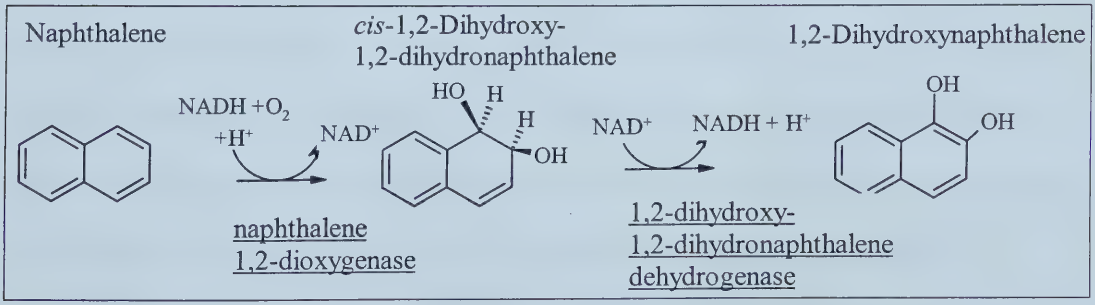
Studies of other aromatic compounds, including toluene, (*p, m*)-xylene, naphthalene, phenanthrene and anthracene, showed that the microbial transformation of aromatic compounds starts with similar oxidation reactions. The oxidation reaction is accomplished either by monooxygenases (hydroxylases) or dioxygenase enzymes. The

former transfers one atom of O_2 into the aromatic structure as a hydroxyl (OH) group and then a second oxygen is added (Madigan et al., 1997). The electron donor for this reaction is the coenzyme NADH or NADPH, as in Equation (2-1) (Rehm & Reed , 1984).

$$R - H + NADH + H^{+} + O_2 \rightarrow R - OH + NAD^{+} + H_2O \qquad \textbf{(2-1)}$$

The most important reaction in the bacterial degradation of aromatic compounds is the *cis*-dihydroxylation, where both atoms of the oxygen molecule are simultaneously incorporated into the aromatic structure and form consistently the *cis*-configuration of the dihydrodiol. The dihydrodiol is then dehydrogenated via dehydrogenase enzymes. As an example, the degradation of naphthalene starts with the formation of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene, then the dehydrogenase enzyme gives 1,2-dihydroxynaphthalene as in Figure 2-8.

Figure 2-8: Oxidation of naphthalene to 1,2-dihydroxy-naphthalene (Rehm & Reed 1984)


Naphthalene
cis-1,2-Dihydroxy-1,2-dihydronaphthalene
1,2-Dihydroxynaphthalene
NADH +O2 +H+
NAD+
HO
H
H
OH
NAD+
NADH + H+
OH
OH
naphthalene 1,2-dioxygenase
1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase

From the dihydrodiol intermediate, the structure undergoes ring cleavage either through the intradiol pathway or the extradiol pathway depending on the microorganism,

the type of the aromatic compound and on the substrate used for growth (Rehm & Reed, 1984). The intradiol enzymes cleave the ring between the two hydroxyl groups, and the extradiol enzymes cleave the ring between one hydroxylated carbon and its adjacent nonhydroxylated carbon. With these basic steps, the following sections will consider two examples of PAC biotransformation; one for naphthalene and the other for dibenzothiophene.

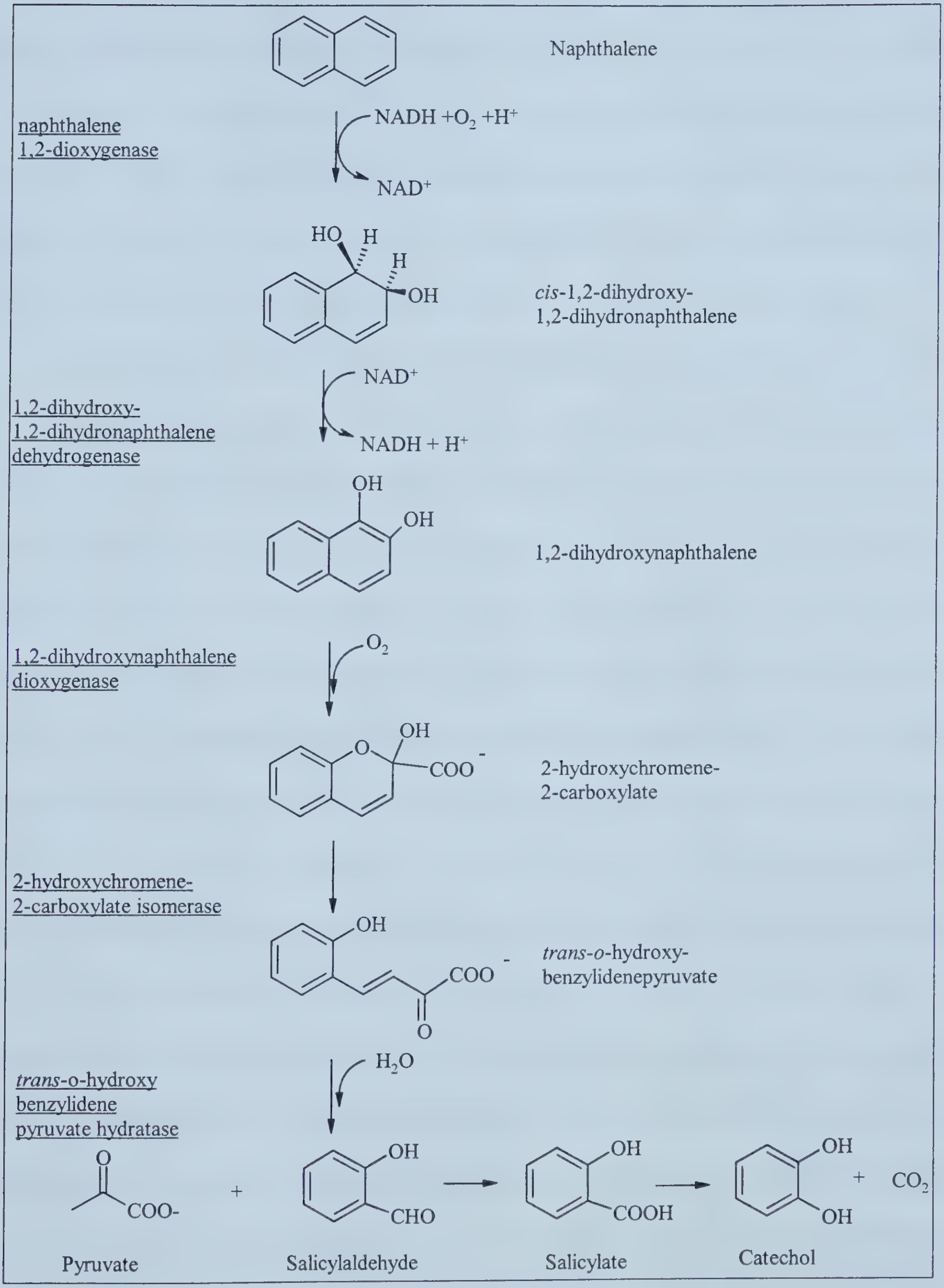
2.3.2.1 Naphthalene Biotransformation

Naphthalene is the simplest fused PAC. Therefore, information obtained from studies of different bacterial strains degrading naphthalene is valuable for understanding and predicting the pathways used in the metabolism of more complex PACs and related heterocyclic aromatic compounds (Eaton & Chapman, 1992). The majority of reports indicate that the genes for utilization of naphthalene as a sole carbon source are plasmid encoded (Silver et al., 1990; Neidhardt et al., 1990). One of these plasmids is NAH7, which encodes the pathway for metabolism of naphthalene via salicylate. NAH7 localizes many naphthalene degradation genes indicated as (*nah*); they are organized in two coordinately controlled operons: *nah-1* and *nah-2*. The first operon (*nah-1*) encodes the oxidation of naphthalene to a salicylate, which is referred to as the upper naphthalene catabolic pathway. The second operon (*nah-2*) encodes enzymes that convert salicylate to catechol, and to its further metabolism via the extradiol cleavage pathway (Harayama & Rekik, 1989, Silver et al., 1990, Eaton & Chapman, 1992, Denome et al., 1993).

A detailed naphthalene degradation pathway is presented in Figure 2-9 for a *Pseudomonas* strain (Denome et al., 1993). Naphthalene is first oxidized via a

dioxygenase enzyme to *cis*-1,2-dihydroxy-1,2-dihydronaphthalene, which is then dehydrogenated to 1,2-dihydroxynaphthalene via a dehydrogenase enzyme. The dihydrodiol is ready for *meta* ring cleavage to form *trans-o*-hydroxybenzylidenepyruvate, and then to salicylaldehyde. The second operon (nah-2) takes over the rest of the degradation pathway.

Figure 2-9: Naphthalene degradation pathway (Denome et al., 1993)


Naphthalene
naphthalene 1,2-dioxygenase
NADH +O2 +H+
NAD+
HO H
H
OH
cis-1,2-dihydroxy-1,2-dihydronaphthalene
1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase
NAD+
NADH + H+
OH
OH
1,2-dihydroxynaphthalene
1,2-dihydroxynaphthalene dioxygenase
O2
OH
O
COO-
2-hydroxychromene-2-carboxylate
2-hydroxychromene-2-carboxylate isomerase
OH
COO-
O
trans-o-hydroxy-benzylidenepyruvate
trans-o-hydroxy benzylidene pyruvate hydratase
H2O
O
COO-
+
OH
CHO
OH
COOH
OH
OH
+
CO2
Pyruvate
Salicylaldehyde
Salicylate
Catechol

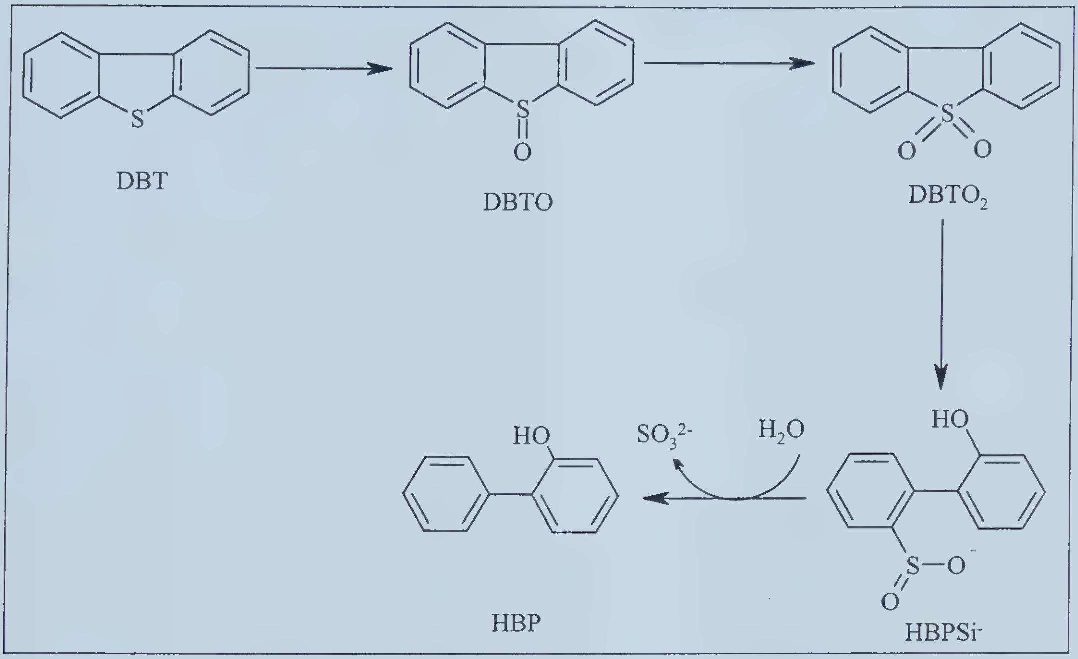
2.3.2.2 Dibenzothiophene Biotransformation

Dibenzothiophene (DBT) is metabolized aerobically by different bacterial strains through different biotransformation mechanisms. DBT can be used as a sole sulfur source, or as a carbon and energy source, or it can oxidized without being an energy source (i.e. cometabolized). The former metabolism is referred to as DBT desulfurization where the other two are referred to as DBT degradation. There are practical usages for both biotransformation mechanisms (Monticello, 1993; McFarland et al., 1998).

In refineries, the conventional hydrodesulfurization process is the primary method for removing organic sulfur compounds from petroleum streams. However, it leaves complex forms as DBT and related substituted compounds (Takatsuka et al., 1997). One possible alternative is using microorganisms with capabilities to selectively remove the sulfur atom. Several microorganisms were found to desulfurize DBT, forming a sulfur-free compound, 2-hydroxybiphenyl (HBP) without assimilating the carbon (Ohshiro & Izumi, 1999). The best characterized bacterium for desulfurization is *Rhodococcus erythropolis* IGTS8, which utilizes DBT as a sole sulfur source from its growth media. One of its desulfurization pathways is shown in Figure 2-10. The first enzyme is a monooxygenase that sequentially incorporates two oxygen atoms. The first atom converts DBT to dibenzothiophene 5-oxide (DBTO), and the second converts DBTO to dibenzothiophene 5,5-dioxide ($DBTO_2$). The second enzyme is also a monooxygenase which transfers $DBTO_2$ to 2-(2'-hydroxyphenyl)benzene sulfinate (HBPSi). Finally, the third enzyme will transforms HBPSi by removing a sulfur group to form HBP and sulfite (Oldfield et al., 1997). Other bacterial strains in the resting state desulfurized DBT in the

presence of high concentrations of hydrocarbons, and with complicated DBT analogs (Ohshiro & Izumi, 1999; Konishi et al., 1997).

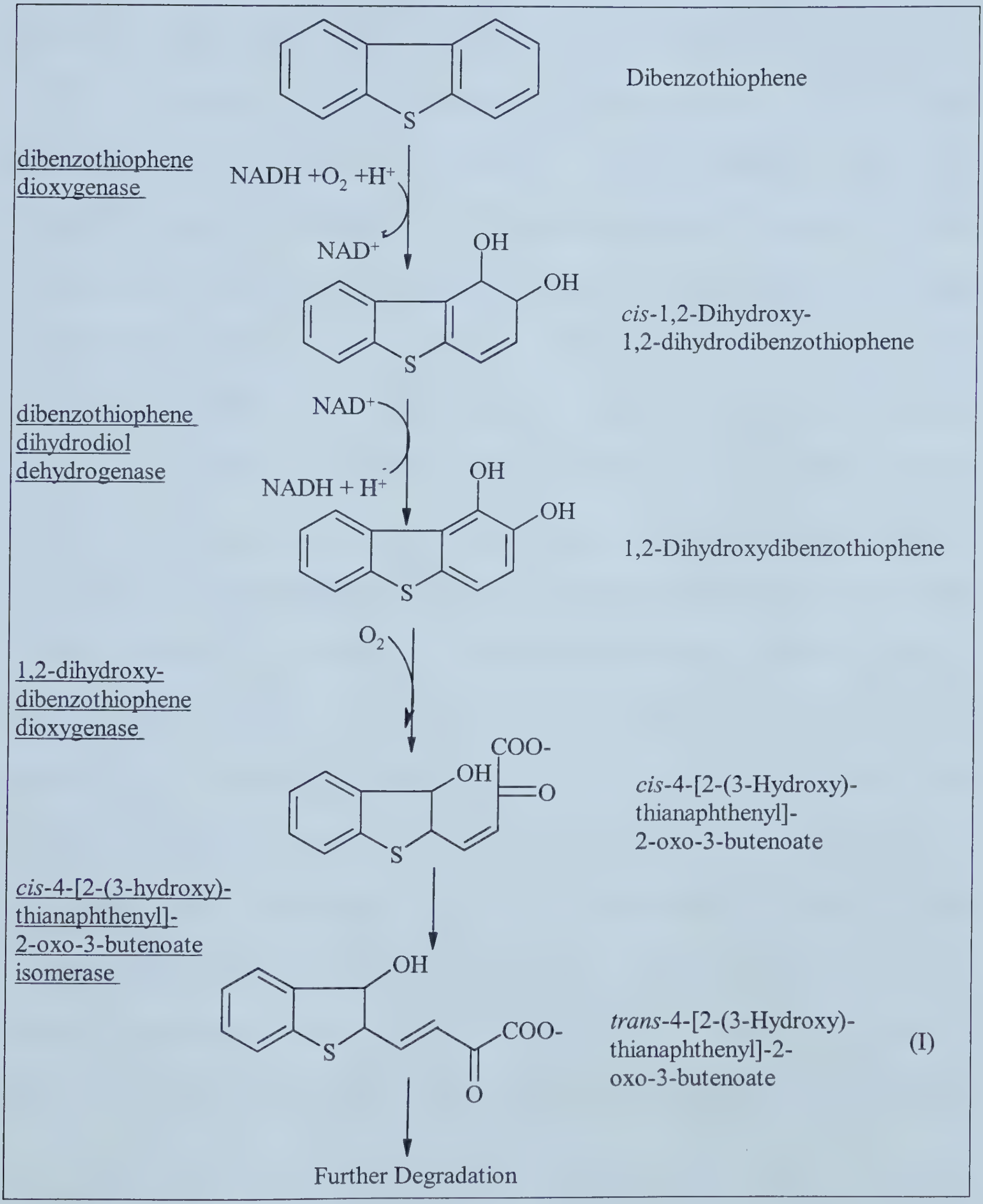
Figure 2-10: ***Rhodococcus erythropolis*** **IGTS8 desulfurization pathway**



The second biotransformation mechanism of DBT depends on the carbon skeleton being partially oxidized and cleaved, while keeping the carbon-sulfur bond intact. This degradation mechanism was described by Kodama et al. (1973), where the metabolism pathway of DBT by some *Pseudomonas* strains resulted in the cleavage of one of the aromatic rings and the formation of *trans*-4-[2-(3-hydroxy)-thianaphtheny]-2-oxo-3-butenoic acid (HTOB), as shown in Figure 2-11 (Kodama et al., 1973). This product has an absorption spectrum in the visible range (λ_{max} = 475nm) (Moticello et al., 1985) and is

further degraded to 3-hydroxy-2-formylbenzothiophene (HFBT), which can be mineralized by mixed bacterial cultures (Mormile & Atlas, 1988; Bressler & Fedorak, 2001).

Figure 2-11: Dibenzothiophene degradation pathway (Kodama et al., 1973)


Dibenzothiophene
dibenzothiophene dioxygenase
NADH +O$_2$ +H$^+$
NAD$^+$
OH
OH
S
cis-1,2-Dihydroxy-1,2-dihydrodibenzothiophene
dibenzothiophene dihydrodiol dehydrogenase
NAD$^+$
NADH + H$^+$
1,2-Dihydroxydibenzothiophene
O$_2$
1,2-dihydroxy-dibenzothiophene dioxygenase
COO-
=O
cis-4-[2-(3-Hydroxy)-thianaphthenyl]-2-oxo-3-butenoate
cis-4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoate isomerase
trans-4-[2-(3-Hydroxy)-thianaphthenyl]-2-oxo-3-butenoate
(I)
O
Further Degradation

The Kodama pathway has shown biochemical similarities with the oxidative pathway of naphthalene, phenanthrene, and anthracene (Harayama & Rekik, 1989; Denome et al. 1993). Furthermore, recent studies suggested that the regulatory genes for degrading certain aromatic compounds are very similar to one another (Silver et al., 1990). In one study, a soil isolate, identified as *Pseudomonas* sp. C18, was able to metabolize DBT to UV-fluorescent metabolites. From this isolate, a 9.8-kb DNA fragment was isolated and transferred to *Pseudomonas putida* and *Escherichia coli* where it exhibited DBT metabolism. This group of genes was designated as DOX, which stands for DBT oxidation. Moreover the nucleotide sequence showed that many of the *dox* open reading frames (ORFs) had sequences similar to genes for enzymes degrading other PACs, such as naphthalene, biphenyl, benzene, and toluene dioxygenases. The upper naphthalene catabolic pathway genes (*nah-1*) had a genetic order identical to the one for DOX genes, with the exception of one gene. Moreover, the DOX enzymes were able to degrade naphthalene to salicylate, yet they did not show any similarities with the sequence from the salicylate operon, which meant that the DOX sequence encoded a complete upper naphthalene catabolic pathway, and indicated that a single genetic pathway controls the metabolism of DBT, naphthalene, and phenanthrene in *Pseudomonas* strain C18 (Denome et al., 1993). Stringfellow and Aitken (1995) with *Pseudomonas stutzeri* P-16 and *Pseudomonas saccharophila* P-15 showed that enzymes involved in the transformation of one PAC could interact with other PACs. However, further studies are needed to follow these studies in investigating other bacterial strains.

2.4 *Pseudomonas* Biocatalyst

Since the late 1950's, several *Pseudomonas* strains have been recognized for their abilities to degrade a wide range of PACs. The PACs were either utilized as a sole carbon source or were cometabolized. Evans et al. (1965) isolated D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene from a soil *Pseudomonas* growing on naphthalene when $NADH_2$ was provided. Subsequent results obtained by Jeffrey et al. (1975) with *Pseudomonas putida* confirmed the bacterial oxidation of several PACs. Since then, several *Pseudomonas* strains showed these oxidative abilities with a variety of compounds (Nakazawa et al., 1996). Consequently, strains of *Pseudomonas* are an excellent potential source of interesting biocatalytic activity toward PACs.

2.4.1 Strain LP6a Isolation

Foght and Westlake (1996) isolated bacterial strain *Pseudomonas fluorescens* LP6a from a mixed enrichment culture derived from a petroleum condensate-contaminated soil. The wild type was selected for further study due to its ability to form oxidized colored metabolites from dibenzothiophene. Upon purification, the strain utilized other PACs, including naphthalene, anthracene, phenanthrene and 2-methylnaphthalene as a sole carbon and energy sources. In addition, it co-metabolically oxidized other PACs such as fluorene, biphenyl, acenaphthene, 1-methylnaphthalene, indole, benzothiophene, dibenzothiophene and dibenzofuran (Foght & Westlake, 1996). Some of these compounds are shown in Figure 2-12. Later, growth of LP6a on PACs was

compared with several bacterial strains that had similar degradative abilities and some of these results are presented in Table 2-2 (Foght & Westlake, 1996). The growth was determined on mineral medium agar plates and compared with inoculated agar without adding the test substrate.

Figure 2-12: Polynuclear aromatic compounds degraded by strain *Pseudomonas fluorescens* LP6a

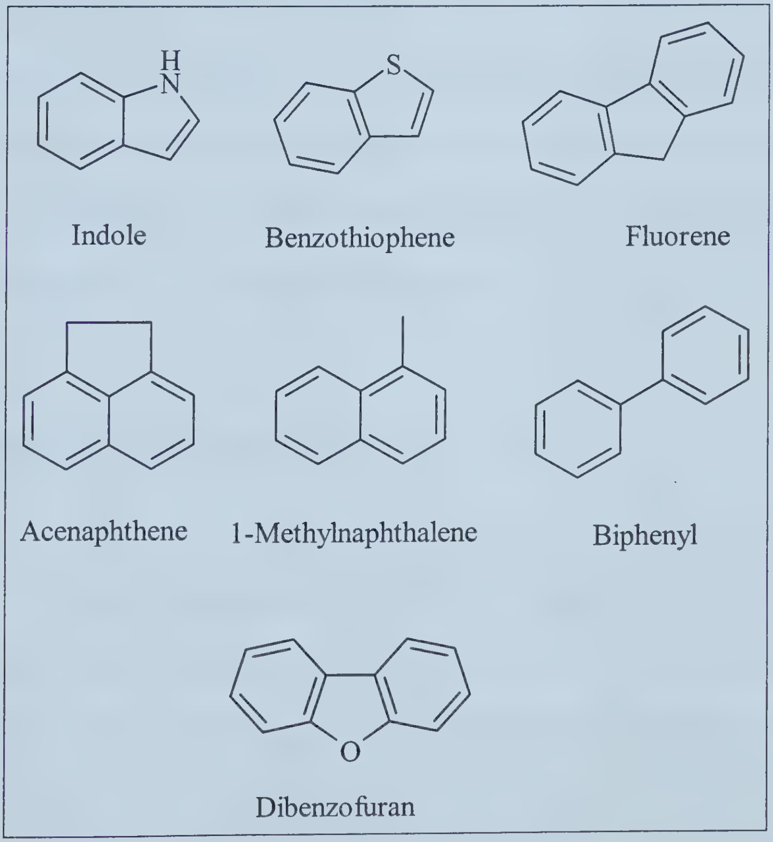

H
N
Indole
S
Benzothiophene
Fluorene
Acenaphthene
1-Methylnaphthalene
Biphenyl
O
Dibenzofuran

Table 2-2: Growth of bacterial isolates on aromatic compounds (Foght &Westlake, 1996)

Bacterial Strain	Growth			
	Naphthalene	Salicylic acid	Phenanthrene	Anthracene
Pseudomonas fluorescens LP6a (pLP6a)	++	++	++	++
P. putida 1064 (NAH7)	++	++	-	-
P. putida NCIB 9816-3 (pWW60, pWW61)	++	++		-
P. paucimobilis WW3	-	-	++	+
Mycobacterium sp. RJGII134	-	-	++	++

Growth determined on Mineral Medium agar: (++) good growth; (+) poor or slow growth; (-) no growth, compared with inoculated agar without added substrate.

The strain LP6a was able to grow on naphthalene, phenanthrene, anthracene, and the metabolite salicylic acid. This behavior was unusual, because most of the other strains grew either on naphthalene or phenanthrene, but not both (Foght & Westlake, 1996). These comparisons were carried further to the genetic level.

2.4.2 LP6a Transformation of PACs

The wild type of *Pseudomonas fluorescens* LP6a has a single plasmid (pLP6a) estimated at 63 kbp that encodes the enzymes for PAC degradation. The plasmid pLP6a had a restriction pattern significantly different from the other plasmids in the bacteria listed in Table 2-2, even from the naphthalene-degradative plasmid NAH7 from *P. putida* 1064, and pWW60 from *P. putida* NCIB 9816-3. However, the hybridization of purified pLP6a with the other plasmids revealed major homology between pLP6a and the naphthalene-degradative plasmids NAH7 and pWW60 in the degradative and non-degradative regions. There was no hybridization of pLP6a with the plasmids from the

other aromatic-degrading bacteria that showed positive growth on phenanthrene and anthracene.

Later, several mutants of LP6a were generated by random insertion of transposon *Tn5* into the plasmid, which added an antibiotic resistance gene for kanamycin. Some mutants lost the ability to grow on naphthalene; others lost the dioxygenase activity, or the ability to utilize salicylate. There was difficulty in obtaining a cured mutant free of the plasmid nevertheless it was achieved. This observation could indicate that an essential gene was present in the non-degradative region (Foght & Westlake, 1996).

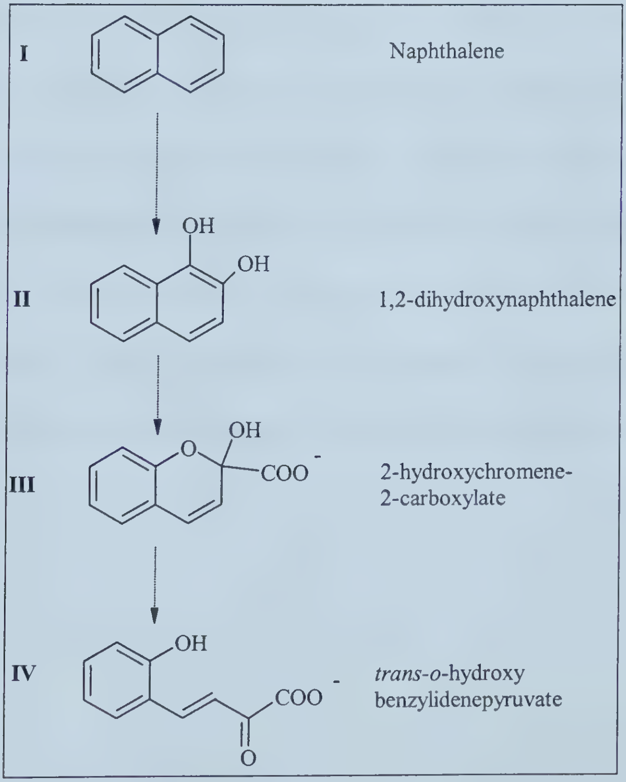
2.4.3 Mutant #21-41 Proposed Biotransformation Pathway

One of the *Tn5* insertions resulted in a mutant referred to as mutant #21-41 and its oxidizing enzymes were induced by salicylic acid and 2-aminobenzoic acid. The mutant displayed a blocked degradation pathway of naphthalene, phenanthrene, alkylated naphthalene and dibenzothiophene. The proposed blocked degradation pathway of naphthalene is shown in Figure 2-13, where the open-ring metabolite *trans*-o-hydroxy-benzylidenepyruvate (IV) is the major accumulated end product. In the case of DBT the equivalent metabolite end product would be *trans*- 4- [2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenate (structure I in Figure 2-11).

The blocked biotransformation capability of this mutant is attractive for modifying the quality of Alberta diesel fuel, since the local produced diesel fuel from bitumen has more naphthenes and substituted naphthalenes than the conventional diesel fuel. If the fused ring structure is opened, and then hydrogenated, it will form an alkylated aromatic compound with higher CN. The proposed biocatalyst system consists

of a sufficient biomass of the mutant, where the chemical and physical conditions are optimized to achieve maximum enzyme activity. The stream of diesel fuel would be introduced to the induced biocatalyst, and allowed to react. The product stream would then be collected and purified, and the biocatalyst recycled. Clearly, the activity of the biocatalyst and its regeneration is a major issue for such a process.

Figure 2-13: Mutant #21-41 blocked degradation pathway of naphthalene (Wu & Foght, 1997)

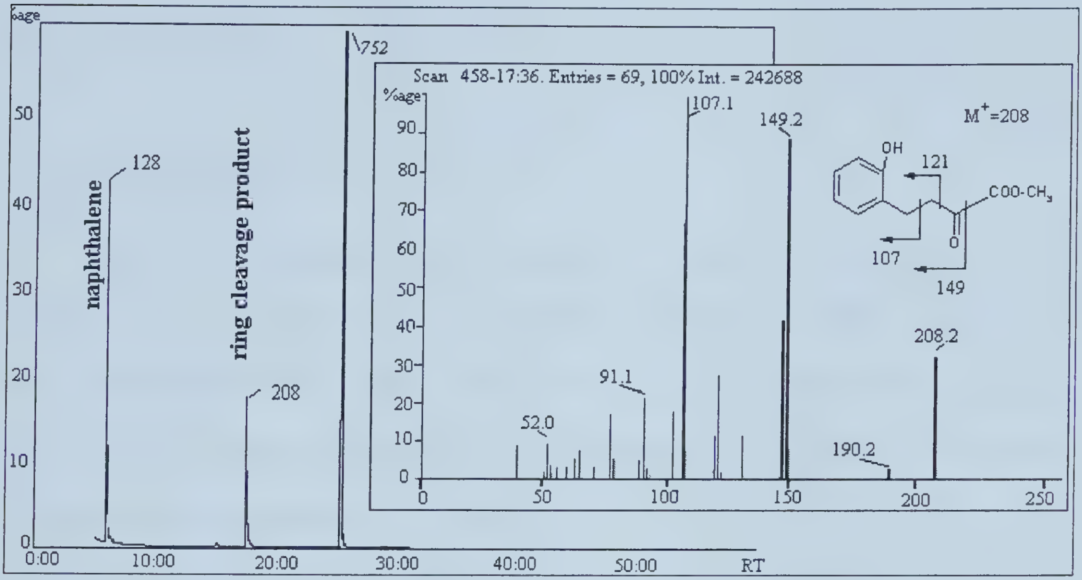

I
Naphthalene
OH
OH
II
1,2-dihydroxynaphthalene
OH
O
COO -
III
2-hydroxychromene-2-carboxylate
OH
IV
COO -
O
trans-o-hydroxy benzylidenepyruvate

2.4.4 Optimization of Biocatalyst Preparation

The selected growth conditions to optimize the biocatalyst activity were as follows: (i) the culture age was 24 hr; (ii) the optimum temp was 28 °C; (iii) the optimum mixing speed was 200 rpm; (iv) the sequence of inducers was salicylic acid (0.5 mM) for 4 hr in growth media, and 2-aminobenzoic acid (0.5 mM) during biocaltalysis (Wu & Foght, 1997).

Subsequently several experiments were conducted to identify the metabolites using different substrates. One of the experiments was a 24 hr incubation of naphthalene with the mutant in which the culture supernatant was acidified and extracted with dichloromethane (DCM), and the samples were further prepared by diazomethane-derivatization for high-resolution gas chromatography mass spectrometry (GC-MS). The ring cleavage products were detected, but the actual isomeric form was not specified, as illustrated in Figure 2-14. The same experiment was done using different alkylnaphthalene compounds where mutant 21-41 generated similar results (Wu & Foght, 1997).

Figure 2-14: Mass spectrum of the ring cleavage product of naphthalene (Wu & Foght, 1997)



After optimizing the biocatalyst conditions and after identifying the open ring metabolites from the different PACs, it is essential to identify means by which the biocatalyst can be recycled and the extent of usability. There are several factors to be considered for biocatalyst recycling, including exhaustion of the cell energy pool (i.e. ATP) and the role of accumulation of potentially inhibitory metabolites.

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganisms

The main microorganism for this study was *Pseudomonas fluorescens* LP6a (mutant #21-41), selected due to its distinctive degradation abilities. *Acinetobacter calcoaceticus* strain RAG-1 (ATCC 31012) and *A. calcoaceticus* strain BD 413 tryp E 27 were used as positive controls in the experiment for measuring cell-surface hydrophobicity (Rosenberg et al., 1980b).

3.1.2 The Media and Buffer

The medium used for growing the microorganisms was Tryptic Soy Broth (TSB) from Difco laboratories (Detroit, USA) prepared as instructed by the manufacturer. The cells were maintained on plates made of Plate Count Agar (PCA) medium prepared using the manufacturer's instructions.

3.1.3 Hydrocarbons

The model compound dibenzothiophene (purity ≥98%) from Fluka Chemical (Switzerland) and was dissolved in hexadecane with (99% purity) from Aldrich Chemical

Company (Milwaukee, USA). The internal standards were biphenyl from Eastman Kodak Company (Rochester, USA) and benzothiophene (thianaphthene) (purity 97%) from Aldrich. Both internal standard compounds were dissolved in hexane with purity (99%) from Fisher Scientific (New Jersey, USA). For liquid-liquid extraction, Pentane with HPLC grade was used (99% purity) obtained from Caledon (Ontario, Canada). For the hydrophobicity tests, xylene was obtained from Fisher and octane from General Intermediates of Canada (Alberta).

3.1.4 Antibiotic and Inducers

Two inducers were used, one during growth and one with resting cells: The first was salicylic acid (SA) (99.9% purity) from Analar the British Drug House LTD (England) dissolved in 95% ethanol, and the second inducer was 2-aminobenzoic acid (2AB) (99-100.5% purity) from British Drug House LTD which was dissolved in N,N-dimethyl formamide (DMF) (99.95% purity) from Sigma. The LP6a mutant #21-41 had kanamycin antibiotic incorporated in all media to a final concentration of (50 mg/L). The antibiotic was from Sigma Chemical Company (St. Louis, MO, USA).

3.1.5 Other Compounds

For the rejuvenating experiments the chemicals used were D-glucose from BDH (Toronto, Canada), ascorbic acid (99% purity) from BDH, and dithiothreitol (DTT) (purity >99%) from Sigma. The drying agent was anhydrous sodium sulphate from BDH.

3.2 Methods

3.2.1 Microbial Growth

The LP6a strain was maintained on plate count agar (PCA) plates with 50 mg/L kanamycin to avoid contamination and to maintain the transposon insertion in the plasmid. Fresh plates were made every two weeks from –70 °C glycerol stock. The streaked plates were incubated at 30 °C for 2 days to obtain sufficient growth. The plates were then stored at 4 °C. The other cultures were transferred from glycerol stocks onto a regular PCA plate then incubated at 30 °C, and later stored at 4 °C.

3.2.2 Biocatalyst Preparation

Preparation of 200 mL of biocatalyst was started with a two loops full of culture inoculated into 5 mL tryptic soy broth (TSB) medium with 50 mg/L kanamycin using test tubes (18×100). The cultures were left to grow overnight on a tube roller at 28 °C. Of that culture 2 mL was transferred into a 200 mL TSB sterilized medium, and incubated for another 24 hr at 28 °C on a rotary shaker set at 200 rpm. After 24 hr the first inducer SA was added to a final concentration of 0.5 mM. The culture was kept for 4 hr on the rotary shaker at 200 rpm. Selecting SA as the first inducer had several advantages: SA is natural, efficient inducer of the system, and is metabolized by the biocatalyst during incubation so that it does not interfere with subsequent chemical analysis of ring cleavage metabolites.

The induced cells were centrifuged in 250 mL centrifuge bottles at 16,300xg for 10 min at –4 °C. They were washed once with 100 mL (0.1 mM) phosphate buffer (pH 7). The pellets were then suspended in 200 mL of buffer, where the second inducer 2-aminobenzoic acid was added to a final concentration of 0.5 mM. The addition of the second inducer was required because SA is depleted during incubation with the substrate, resulting in declining activity of ring cleavage during the substrate biotransformation. However, 2-aminobenzoate (2AB) is an artificial inducer of the biocatalyst and it was not metabolized. Thus, it is present throughout the incubation period to maintain induction of the biocatalyst. The substrate DBT in not known to be an inducer of biocatalysis in mutant #21-41.

Once the biocatalyst was induced, it was ready to receive the substrate dissolved in an organic carrier. The organic phase (hexadecane + DBT) was added to the aqueous phase with a volume ratio of 1:100. The substrate DBT was dissolved in the organic phase of hexadecane to give stock solutions with concentrations from 0.1 M to 0.25 M. Table 3-1 presents the variation in chemical volume as the biocatalyst volume was changed. The final DBT concentration on a total volume basis in the biocatalyst suspension varied from 1 mmole/L to 2.5 mmole/L. At any given time, DBT would partition between the hydrocarbon phase, the aqueous phase, and the cells. Details of stock solution preparation are explained in **Appendix B.**

Table 3-1: Variation in chemical volume as the biocatalyst volume was changed

Biocatalyst liquid volume (mL)	100	200	400	500	1000	2000
Tryptic soy broth media (g)	3	6	12	15	30	60
Kanamycin antibiotic (mL)	0.2	0.4	0.8	1	2	4
Salicylic acid (0.5 M) (mL)	0.1	0.2	0.4	0.5	1	2
2-Amino benzoic acid (0.5 M) (mL)	0.1	0.2	0.4	0.5	1	2
DBT (0.1 M) (mL)	1	2	4	5	10	20

3.2.3 Biocatalyst Analysis

Several analyses were performed on the biocatalyst to determine the physical characteristic of the cells during growth. All the measurements were done on samples of resting cells in buffer unless mentioned otherwise.

3.2.3.1 *Optical Density (OD_{600})*

Three or more 100 µL or 50 µL samples were diluted with doubly distilled water (ddH_2O) to a volume of 1 mL. The spectrophotometer was set to 600 nm and blanked with ddH_2O. The measured absorbance readings were maintained within the linear range, which was less than 0.5 absorbance.

3.2.3.2 Dry Weight

A set of three 30 mL samples of biocatalyst suspension was collected and centrifuged for 16 min at a speed of 13,000xg. Each sample pellet was then resuspended in 5 mL buffer and spread on a dry aluminum plate of known weight. The blank was a 5 mL buffer sample dried at the same time interval of 5 days in a 70 °C oven. Later the dry

samples were weighed and the weight of the blank was subtracted to account for the weight of salts in the buffer.

3.2.3.3 Viable Count

A series of 10-fold dilutions of a 1 mL sample were made until a dilution of 10^{-6} and/or 10^{-7} was reached. From each dilution, 0.1 mL was spread onto plate count agar plates containing kanamycin (50 mg/L) and incubated at 30 °C for 24 hr. One plate was prepared for each dilution until 10^{-5} dilution was reached then three plates were prepared. Any plate with less than 30 or more than 300 colonies was not considered.

3.2.3.4 Protein Assay

The protein content of the biocatalyst was measured using the microplate bicinchoninic (BCA) assay kit. Triplicate samples of 1 mL each were taken from the biocatalyst. A 100 µL, 50 µL or 20 µL volume was used for analysis of protein content in the biocatalyst. Each sample was centrifuged for 10 min at 14,000 rpm, the spent buffer was removed, and 0.1 mL NaOH (1 N) was added. The samples were then left for 30 min in a 60 °C water bath, where the cells lysed. Meanwhile, the BCA reagents A and B were prepared in a ratio of 1:50 of A:B. Ten microliters of each sample were added to three assigned wells in the microplate, another three wells each had 10 µL of the blank 0.1 M NaOH, and another three had 10 µL of the standard solution of bovine serum albumin (BSA) from the kit. Each concentration was represented in three wells, starting with 2000 µg protein/mL to 20 µg protein/mL. Later, 0.2 mL of the reagent was added to each well and left for another 30 min at 60 °C in a water bath. Finally, the microplate was cooled to room temperature and the color intensity was read at an absorbance of 570 nm. The

standard concentration was linear in the given range and the samples were diluted to ensure the accuracy of the results.

3.3 Chemical Analysis

3.3.1 Dibenzothiophene

In this work the desired open ring metabolites from DBT will be referred to as the end product, *trans*-4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid (Kodama et al., 1973; Monticello et al., 1985). To stop the activity of the biocatalyst, 6 N HCl was added to drop the pH below 2. For a 200 mL biocatalyst volume, about 1.3 mL acid was added. The sample was stored at 4 °C overnight. The biocatalyst was brought to neutral pH by adding about 0.8 mL of a 10 N NaOH solution. The unconverted DBT in the organic phase was then recovered by liquid-liquid extraction using pentane. The separatory setup consisted of 500 mL or 60 mL separation funnels depending on the biocatalyst volume of 200 mL or 30 mL, respectively. After transferring the biocatalyst to the separatory funnel, about 50 mL pentane was added and the Erlenmeyer flask was washed with pentane to recover all of the organic phase. The process was repeated 3 to 4 times for a 200 ml total approximate volume of pentane. The pentane phase was passed through the drying agent sodium sulphate (anhydrous granular).

The collected dried pentane was evaporated at ≈ 35 °C using a rotary evaporator (model Rotavapor R-112, Buchi, Switzerland) immersed in a water bath (model waterbath B-480, Buchi). The solvent-free organic phase sample was transferred to an 8 mL vial. Two methods were used for preparing the samples for GC. The first was the addition of 100 μL of the sample, with 50 μL of internal standard (IS) and 900 μL of

dichloromethane (DCM) to the GC vials. The second preparation method involved transferring 1 mL of the sample with the addition of 150 μL internal standard and 4.5 mL DCM. The latter method was more convenient, and it was used for the last interval of the research project. The results from the two methods did not show significant differences. The GC used was a Hewlett Packard 5890A equipped with hydrogen flame ionization detector (USA) and a HP-1 crosslinked methyl silicone gum column. The column dimensions were 25 m long, 0.32 mm inner diameter, and 0.17-μm film thicknesses. The flow rate of helium, the carrier gas, was 19 mL/min. The temperature at the injector and detector was 270 °C. The temperature program was 2 min at 90 °C, incremented by 5 °C/min to 130 °C, then incremented by 1.5 °C for 16 °C and 50 °C/min to 250 °C where it remained for 4 min. The retention times observed were 4.4 min for benzothiophene, 8 min for biphenyl and 20 min for dibenzothiophene. The remaining retention times were 1 min and 17 min for the solvents DCM and hexadecane, respectively.

The biocatalyst activity was characterized by two terms; the absolute DBT conversion and the rate of conversion. The absolute DBT conversion was defined as amount of DBT converted (or consumed) per total protein content in the batch. The rate of conversion was defined as the amount of DBT converted per total protein content per time of reaction. The time of reaction corresponded to the time the DBT sample was drawn from the biocatalyst mixture. The calculations are explained in **Appendix B**.

3.3.2 Measurement of Metabolites

The opened ring metabolite of DBT degradation is water soluble with brownish red color (Kodama et al., 1973) and has maximum in its absorption spectrum of 475 nm

(Monticello et al., 1985). After adding the substrate, at timed intervals 1 mL samples were taken from the aqueous phase of the biocatalyst. The samples were centrifuged in a micorfuge for 2 min to separate the pellets from the buffer. Supernatant samples of volumes 20-50 or 100 μL were taken in triplicates and diluted to 1 mL with ddH_2O. After blanking the spectrophotometer with ddH_2O, the samples were read at 475 nm, ensuring that absorbance readings were below 0.3 absorbance units.

3.4 Analysis of DBT Metabolites from LP6a Mutant 21-41

The open ring products of DBT were polar compounds that could not be directly detected by GC-FID or extracted using the same procedure as DBT. These products required derivatization to be identified. Thus the metabolites were extracted, derivatized with diazomethane and then analyzed using gas chromatography mass spectrometry (GC-MS).

A 700 mL batch of induced biocatalyst was prepared then divided and resuspended in 2x200 mL volume batches. The two batches each had 2 mL (0.1 M) DBT in hexadecane, and no 2AB was added since it interferes with GC analysis. The first batch was terminated after 4 hr and the second after the 16th hr. The batches were terminated by adding 3x100 mL of pentane. The solvent layer was drained without passing through a drying agent, to avoid loss of any unknown metabolites, then the solvent was evaporated and the sample was derivatized by diazomethane. The sample from this step was referred to as sample A.

The cell pellets were removed by centrifugation at 16,300xg for 10 min. The remaining aqueous phase was acidified by 1.3 mL (6 N) HCl to pH = 2. Then

approximately 3x100 mL of dichloromethane (DCM) was added to the aqueous phase, where the solvent layer was collected without passing through a drying agent, and the sample free of solvent was derivatized with diazomethane. The sample from this step was referred to as sample B. The aqueous phase was acidified to a pH less than 2, and the same procedure was followed to collect the derivatized sample C.

The samples were analyzed using a low-resolution GC-MS using a Hewlett Packard 5890 series II gas chromatograph with a 5970 series mass selective detector and a 30 m DB-5 capillary column (J&W Scientific, Folsom, California, USA). The GC temperature program used was 90 °C for 1 min and then an increase of 5 °C/min to 280 °C for 21 min.

3.5 Kinetics of DBT Conversion and Metabolite Formation

3.5.1 During 28 hr Experiment

DBT was used as the model compound mainly because its water-soluble ring cleavage products have an absorption maximum at 475 nm that was readily quantified spectrophotometrically. Therefore we attempted to correlate the color intensity with the conversion of DBT. The finding of such a correlation would permit rapid quantification of ring cleavage without solvent extraction and GC analysis.

Two batches of 800 mL induced biocatalyst suspension were prepared. The biocatalyst volume was divided into 23x250 mL Erlenmeyer flasks each with 30 mL, and 3x500 mL Erlenmeyer flasks each with 200 mL. The 30 mL volume was used for measuring DBT conversion via pentane extraction and GC analysis. The absorbance of

the metabolites at 475 nm was measured by taking 1 mL sample from each 200 mL batch at timed intervals. At each time interval two of the 30 mL batches were terminated by adding 0.2 mL (6 N) HCl, which was stored at 4 °C over night. The addition of 0.12 mL (10 N) NaOH neutralized the 30 mL batches. The duration of the experiment was 28 hr.

3.5.2 During 51 hr Experiment

The design of the following experiment was similar to the previous one, but it was aimed at determining if mass transfer was a limiting factor, and thus two concentrations were tested (1 mmole/L) and (2.5 mmole/L). The higher concentration was close to saturation levels of DBT in the organic phase. The experiment time was extended from 28 hr to 55 hr to further study the changes in conversion and the absorbance of metabolites. The experimental conditions, i.e. incubation temperature at 28 °C, mixing at 200 rpm, and the ratio of organic carrier to the aqueous phase all were kept constant. Thus, the only variable in these tests will be the initial concentration of DBT in hexadecane.

The biocatalyst was prepared in 4 batches each with 700 mL TSB media. After 24 hr a 0.7 mL volume of (0.5 M) salicylic acid was added to each flask, which was shaken for 4 hr and then centrifuged. The collected pellets were resuspended to an OD_{600} of 3.7. The induced biocatalyst was divided into two groups. Group A, in 60x125 mL Erlenmeyer flasks with 30 mL biocatalyst volume was used to measure DBT conversion using solvent extraction and GC analysis. Group B in 4x500 mL Erlenmeyer each with 200 mL volume, was used to collect 2x1 mL samples to measure the absorbance of metabolites. Group A had 0.3 mL of substrate and 30 μL volume of 0.5 M 2AB. Group B

had 2 mL of substrate and 0.2 mL volume of 0.5 M 2AB. Half the flasks had a DBT concentration of 1 mmole/L while the other half had 2.5 mmole/L concentration. At the initial time, two flasks of 30 mL volume from each concentration set were terminated by adding 0.15 mL HCl. A 1 mL sample was taken from the 200 mL volume from each concentration set. The same procedure was carried out until the 55th hour was reached. Adding 0.2 mL of (10 N) NaOH neutralized the acidified flasks prior to pentane extraction.

3.5.3 During a 20 hr Experiment Starting with Two Different Initial Concentrations

This section will study the reaction rate for two concentrations: 1 mmole/L and 2.5 mmole/L, for the first 20 hr. The induced biocatalyst suspension was prepared for a total volume of 2.6 L with an OD_{600} of 3.8. The biocatalyst volume was divided into two sets. Each set had 20x125 mL Erlenmeyer flasks filled with 30 mL of the biocatalyst, and 2x500 mL Erlenmeyer flasks filled with 200 mL. One of the 200 mL batches was spoiled, thus only one batch was used to measure the absorbance of the metabolites for the 1 mmole/L concentration. Three hundred microliters of the specified DBT concentrations in hexadecane were added to the 30 mL volume and 2 mL to the 200 mL volume. The inducer 2AB was added in the same proportions as in previous experiments. The 30 mL volume was used to measure DBT conversion via extraction, and the 200 mL volume was used for monitoring absorbance of metabolites at 475 nm. Starting at the initial time, two flasks of the 30 mL volume from each set were terminated by adding 0.2 mL (6 N) HCl. A 1 mL sample was taken from the 200 mL volume from each set. The

same procedure was carried out at each 2 hr interval, until the 18th hr was reached. Adding 0.12 mL of (10 N) NaOH neutralized the acidified flasks.

3.6 Reuse and Rejuvenation of the Biocatalyst

Since the biocatalyst in this study used cells in the resting state, rejuvenation experiments became one of the research objectives. Before proceeding with rejuvenation, it was necessary to find the appropriate sequence for adding the energy sources. The first attempt was to add the energy source at the initial time.

3.6.1 Simultaneous Supply of Glucose and DBT

The first approach to keep the biocatalyst activity high was the addition of energy sources at the start of the reaction time, without supplying the entire range of nutrients needed for growth. A 500 mL volume of induced biocatalyst was prepared in a 2 L Erlenmeyer flask. The pellets were collected and washed once with buffer, then were resuspended in 500 mL buffer. The biocatalyst was divided into two 200 mL batches, and 2 mL substrate (0.1 M) and 0.2 mL 2AB were added to each. The initial $OD_{600} = 3.6$ and total protein content = 2.0 E6 μg protein/mL. A sterilized glucose solution was added to one batch to a concentration of 5 g/, equal to the amount added to a culture to grow *Pseudomonas* species (Holt et al., 1994). The batches were incubated at 28 °C and shaken at 200 rpm. At a time interval of 5 to 10 min a 1 mL sample was taken from each batch, and centrifuged in a microfuge at 14,000 rpm for 2 min. The absorbance of the supernatant was read at 475 nm following dilution.

3.6.2 Rejuvenation Experiment with a 48 hr Exhausted Biocatalyst

This experiment was designed to study the rejuvenation of the exhausted biocatalyst, by adding energy sources such as TSB media and glucose, or reducing agents such as dithiothreitol and ascorbic acid. The exposure time was short to suppress the formation of new bacteria.

The rejuvenation experiment was done with a biocatalyst used for 24 hr. The energy sources added were TSB media and glucose. The appropriate amounts of kanamycin were added to the media. A 2 mL inoculum of an overnight 5 mL inoculum was transferred to 200 mL TSB media. The 200 mL batch was incubated at 28 °C for 24 hr at 200 rpm. Later 2 mL inoculum was transferred to 5 independent batches of 200 mL TSB; they were incubated at 28 °C for 24 hr at 200 rpm. Salicylic acid was added for 4 hrs. The batches were centrifuged and washed. The collected pellets were resuspended in 1.5 L buffer, and the initial OD_{600} was 3.3. The biocatalyst was divided into 7 batches each with 200 mL; five of them were used to measure conversion of DBT via extraction; and two were used for measuring viability.

Before each 24 hr run a 0.2 mL aliquot of 2AB solution was added. The batches were divided into 3 categories, A, B, and C. Category A defines the biocatalytic activity for the first 24 hr run that was terminated after 24 hr by adding 1.3 mL of 6 N HCl, which dropped the pH to less than 2. Batches in group B were used for 24 hr, and then they were centrifuged independently at 16,300xg for 10 min to collect the pellets and decant the metabolites. The batches were washed with 100 mL buffer, and centrifuged again to collect the pellets. One batch was resuspended in 200 mL buffer. The second was

suspended in full strength (30 mg/mL) TSB medium and the last was suspended in 200 mL buffer with 5 mg/mL glucose. The latter was prepared from an approximation of the total glucose present in the TSB media. The different batches were shaken for 1 hr at 28 °C and 200 rpm. Then they were centrifuged and washed with buffer. The collected pellets were independently resuspended in 200 mL buffer. The batches were shaken for 24 hr at 28 °C and 200 rpm. The batches were terminated with 1.3 mL of 6 N HCl and stored at 4 °C. Next a day the batches were neutralized with 0.8 mL (10 N) NaOH. The remaining DBT was recovered via extraction.

3.6.3 Glucose Consumption Rate for Exhausted Biocatalyst

To monitor the rate of consumption of the energy source, the biocatalyst was used for 24 hr, then different glucose concentrations were added (0.1 mg/mL and 1 mg/mL). A control set of cells was used to compare its rate of consumption with the exhausted biocatalyst. The glucose removal from the biocatalyst medium was tested with glucose Trinder reagent kit (Sigma, USA).

The reagent was tested for its suitability within the concentration range of the current experiment. Therefore a standard glucose solution was tested using the Trinder reagent kit. The glucose Trinder reagent was prepared according to the instructions. The standard solution was diluted using phosphate buffer and the concentration ranged from 0 to 3 mg/mL. Five microliters of the standard solution was added to 1 mL Trinder reagents. The samples were incubated for 18 min at room temperature. The spectrophotometer wavelength was set at 505 nm, were it was zeroed by ddH_2O and the buffer used as the blank. The standard concentration range had a linear relation with 505

nm absorbance with an $R^2 = 0.993$. Thus, the reagent was found to be appropriate for usage in the following experiment.

Two batches each of 450 mL induced biocatalyst were prepared. Each batch was divided into two volume of 200 mL biocatalyst. The four batches were induced with 0.2 mL 2AB and the average $OD_{600} = 4.8$. Two batches had 2 mL (0.1 M) substrate, but not the other two batches. This treatment would show whether the glucose uptake by the biocatalyst was affected by exposure to the substrate. All the batches were shaken for 24 hr at 28 °C and 200 rpm. The batches were centrifuged, washed with buffer, and each was suspended in 200 mL buffer. At this point the average OD_{600} was 3.2. The different glucose concentrations (0.1 or 1 mg/mL) were added, and the glucose disappearance was measured using the glucose Trinder reagent kit. At timed intervals 10 µL of each sample and the blank were added to the reagents. The samples were left for 18 min at room temperature. The spectrophotometer wavelength was set on 505 nm, were it was zeroed by ddH_2O and the buffer used as the blank.

3.6.4 Rejuvenation Experiment with a 72 hr Exhausted Biocatalyst

The current experiment investigated rejuvenation of the biocatalyst after three experimental cycles. Each cycle was for 24 hr, after which the biocatalyst was washed, resuspended in buffer and then the substrate was added. Thus, the duration of the cycles was 48, 72 and 96 hr.

An overnight 5 mL inoculum was prepared as described in the biocatalyst preparation section and 2 mL was transferred to 200 mL TSB medium. This 200 mL culture was referred to as the large seed. The large seed was shaken for 16 hr, at 28°C and

200 rpm. To insure homogeneity and to prepare a large volume of cells, four 2 L flasks were prepared, each containing 600 mL TSB medium. From the large seed a 6 mL volume was added to each 600 mL media. The former flasks were shaken for 24 hr at 28 °C and 200 rpm. On the second day 0.6 mL SA was added to each flask and left for 4 hr. The pellets were collected from the 4 flasks by 10,000-rpm centrifugation for 10 min. After each centrifugation the supernatant was discharged. The pellets were washed with 100 mL buffer and centrifuged by 16,300xg for 10 min, then the supernatant was discharged. The washed pellets were resuspended in a 6 L Erlenmeyer flask using 2500 mL buffer. This cell suspension was referred to as stock biocatalyst. The stock biocatalyst had an OD_{600} of 3.6, total protein content of 1.08 μg protein/mL, and viable count of 5.37×10^9 CFU/mL culture.

The stock biocatalyst volume was divided into twelve 500 Erlenmeyer flask each containing 200 mL. The OD_{600} was measured for each batch, and the average OD_{600} was consistent with the stock biocatalyst optical density. The twelve flasks were labeled from 1 to 12. Flask number 1 (batch #1) was used as a reference to measure viable count and optical density throughout the experimental time. The batch #1 was introduced to the flasks during centrifuging and buffer washing; to monitor the biocatalytic changes due to these steps and no inducer or substrate was added. The remaining flasks had 2 mL of (0.1 M) DBT along with 0.2 mL (0.5 M) 2AB. All the batches were shaken for 24 hr on a rotary shaker at 28°C and 200 rpm. From batch #1 at initial time two 1 mL samples were taken for viable count and protein content measurements.

After 24 hr all the batches were centrifuged in separate 250 mL centrifuge bottles at 16,300xg for 10 min. The metabolite products were decanted and 100 μL samples were

taken to measure the absorbance at 475 nm. The collected pellets were washed with 100 mL buffer, centrifuged at 16,300xg for 10 min and decanted. The washed pellets were resuspended in 200 mL buffer, where 0.2 mL 2AB and 2 mL substrate were added. The batches were placed back on the rotary shaker under the same conditions as the first 24 hr run. From batch #1 three 1 mL samples were taken for viable count, protein content and optical density measurements.

In the second 24 hr run, all the batches were treated similarly as in the first 24 hr run, except two batches were terminated. The termination of two batches was done by adding 1.3 mL (6 N) HCL. These two batches were stored at 4°C for extraction and their results correspond to the second 24 hr biocatalytic activity. The remaining batches were shaken for 24 hr after adding the inducer 2AB and the DBT substrate. From batch #1 3x1 mL samples were taken for viable cell count, protein content and optical density measurements. After the third 24 hr period, one batch was terminated to determine the third cycle biocatalytic activity. From the other nine batches 50 μL volume was taken to measure the metabolite absorbance as described in section 3.3.2 Then the batches were centrifuged and washed with buffer, to remove all the solubilized metabolites and the remaining substrate in the organic phase. The flasks were transferred individually to 200 mL buffer. Six batches had 5 mL of different energy or reducing sources added. The energy source was glucose, while dithiothreitol and ascorbic acid were added reducing agents. Two batches were used as references, therefore no energy source was added. The remaining batch was used for viability and optical density measurements. The flasks were shaken for 80 min at 28 °C, and 200 rpm. Later the batches were centrifuged and washed with buffer. Then a 2 mL substrate and 0.2 mL 2AB were added to all the flasks except

one, and a new 24 hr incubated was conducted. The three 1 mL samples were taken from the DBT-free batch for viable count and OD_{600} measurements. The experiment was terminated after 24 hr by adding 1.3 mL of 6 N HCl acid, and three final samples were taken for viable count and OD_{600}.

3.6.5 Reuse of the Biocatalyst

This experiment was performed where the biocatalyst was reused repeatedly with removal of the DBT metabolites before each stage. Each stage of the experimental runs was for 24 hr and the number of stages were 2, 3 and 4, i.e. 48, 72 and 96 hr total duration, respectively. The experiment contained two independent batches, with each receiving the same treatment.

The appropriate amounts of kanamycin were added to the media. An overnight 5 mL inoculum was prepared as described in the biocatalyst preparation section and 2 mL was transferred to 200 mL TSB medium, referred to as the large seed. The large seed was shaken for 16 hr, at 28°C and 200 rpm. To insure homogeneity and to prepare a large biocatalytic volume, four 2 L flasks were prepared, each containing 600 mL TSB medium. From the large seed a 6 mL volume was added to each 600 mL medium. The flasks were shaken for 24 hr at 28°C and 200 rpm. On the second day 0.6 mL SA was added to each flask and incubated for 4 hr. The pellets were collected from the 4 flasks by 16,300xg centrifugation for 10 min. After each centrifugation the supernatant was discarded. The pellets were washed with 100 mL buffer and centrifuged by 16,300xg for 10 min, where the supernatant was discarded. The washed pellets were resuspended in a 6 L Erlenmeyer flask using 2500 mL buffer. This cell suspension was referred to as stock

biocatalyst. The stock biocatalyst for the both batches had an initial OD_{600} of 3.6 and 3.7, and a total protein content of 1.56×10^6 µg and 1.66×10^6 µg protein.

The stock biocatalyst volume was divided into 9x500 Erlenmeyer flasks, each containing 200 mL. One flask of each batch was used for OD_{600}, protein, and viable counts. The remaining eight flasks each had 2 mL of a 0.1 M DBT substrate and 0.2 mL of 0.5 M 2AB. The batches were incubated at 28°C, shaken for 24 hr at 200 rpm. After each 24 hr period, two flasks from each batch were terminated. One milliliters samples were taken from the remaining flasks for absorbance measurements at 475 nm. The pellets from these flasks were collected and washed with 100 mL buffer, and then resuspended in 200 mL buffer. The appropriate amounts of substrate and inducer were added. Samples were collected from the substrate-free flask to measure the biomass corresponding to the next 24 hr period.

4.0 RESULTS

The objective of this work was to examine the biocatalytic activity in converting dibenzothiophene to an open ring compound. The biocatalyst was whole cells of a transposon mutant of a *Pseudomonas fluorescens* LP6a strain. The mutant was able to co-metabolize DBT to an open ring product, without excessive degradation. The biocatalyst activity was tested under potentially inhibiting conditions, and the activity was monitored with repeated usage of the biocatalyst. A standard procedure for sampling for all measurements was followed to ensure the reproducibility of the data, and large volume samples were collected for quantitative measurements by GC-FID to ensure good repeatability.

4.1 Validation of Experimental Procedures

Before conducting the main experiments and altering the key variables, it was necessary to define and validate the experimental protocols. Therefore, experiments were used to check the following variables: (1) the optimal culture age for harvesting, (2) the cell interaction with the organic phase (hexadecane) and other organic carriers, (3) the viability of the biocatalyst during the course of experiments, (4) the efficiency of extraction of DBT with pentane, (5) the DBT evaporation rates, and finally determining, and (6) the accuracy of the separation and analytical methods.

4.1.1 Growth Characteristics of the Microbial Strains

4.1.1.1 Growth Curve

The information gathered from the growth curves assisted in the design of several further experiments. The three microorganisms used were *P. fluorescens* LP6a (mutant #21-41), *A. calcoaceticus* RAG-1 and *A. calcoaceticus* BD 413. The microbial growth was measured by OD_{600} and protein assay for approximately 5 days. Figure 4-1 illustrates the growth curves of LP6a, RAG-1 and BD 413. This experiment was repeated twice more for strain LP6a. The OD_{600} data are shown in Figure 4-2. The protein content of two LP6a batches is presented in Figure 4-3. Thus, from Figure 4-2 and Figure 4-3 the collection of biocatalyst after 24 hr of growth was during the late exponential – early stationary phase of the culture. The generation time for LP6a was calculated from the slope of the line obtained in Figure 4-4. The calculated average generation time was 1.8 hr.

Figure 4-1: Growth curve of LP6a, RAG-1 and BD 413 based on log OD_{600} versus time

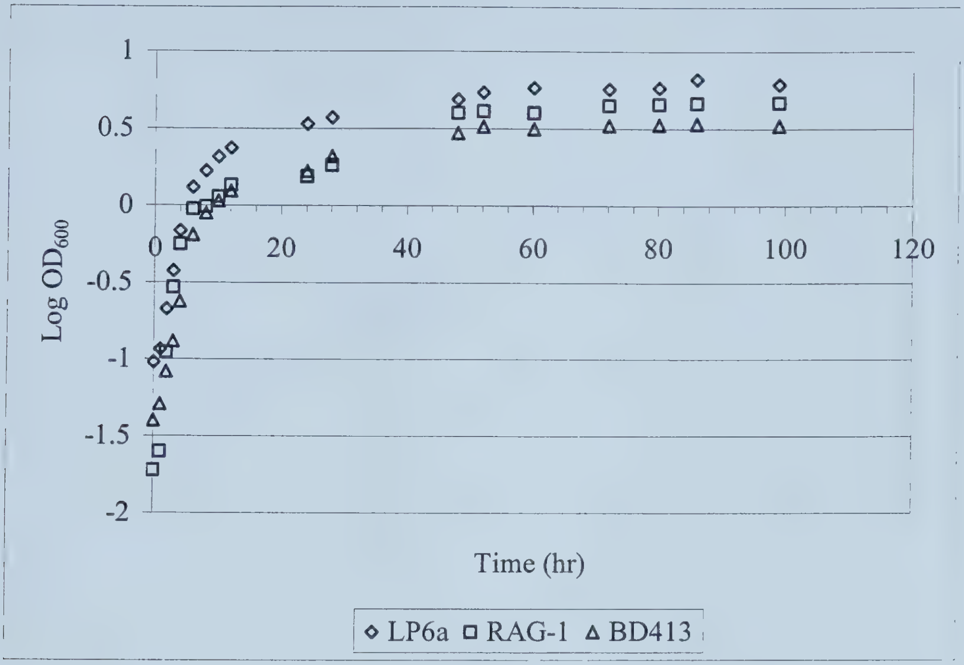


Figure 4-2: Growth curve of LP6a mutant 21-41 based on log OD_{600} versus time for three independent batches

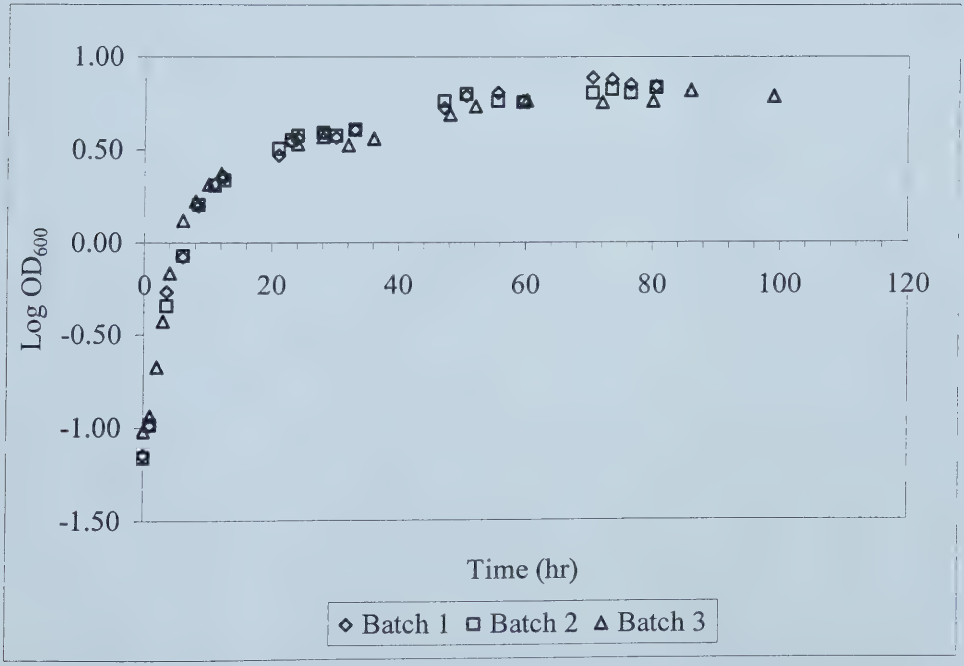


Figure 4-3: Growth curve of LP6a based on total protein content versus time. The data represent the average values of two batches, batch 2 and 3

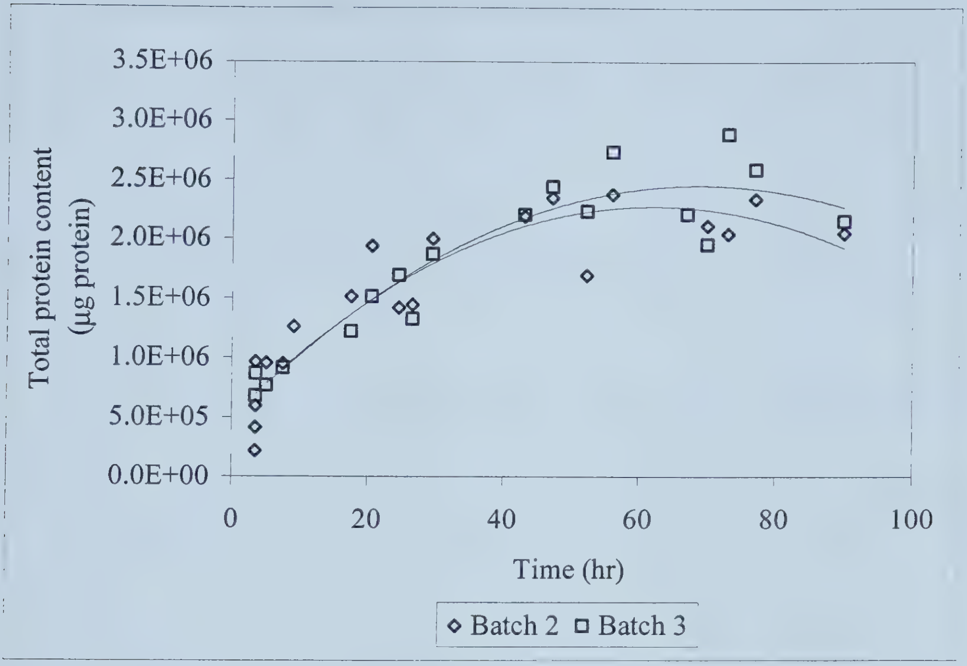
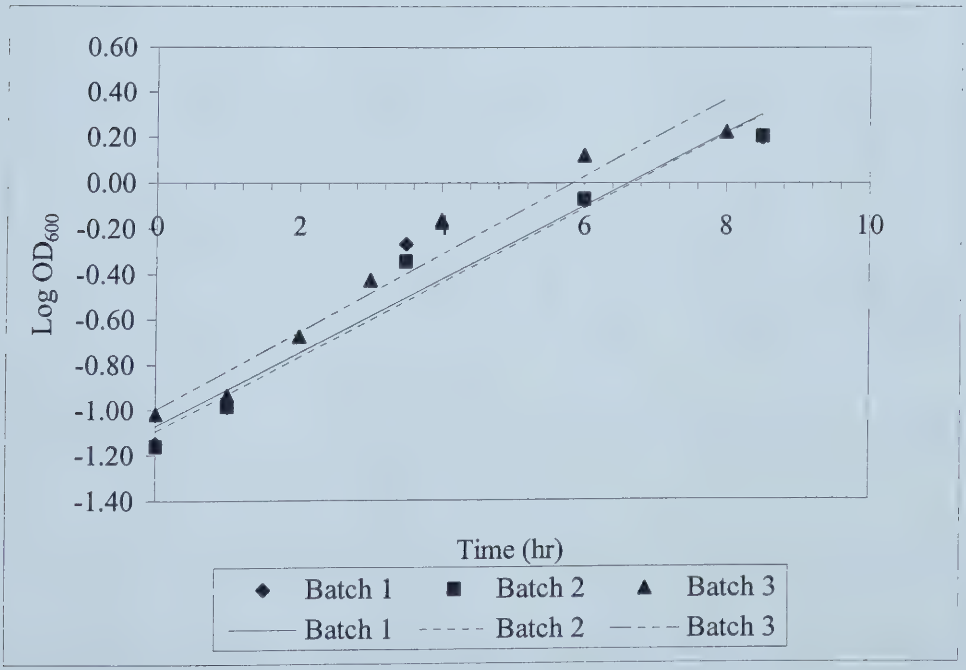


Figure 4-4: Growth of LP6a mutant #21-41 during the exponential growth phase. The Lines show linear regression to the data from each batch

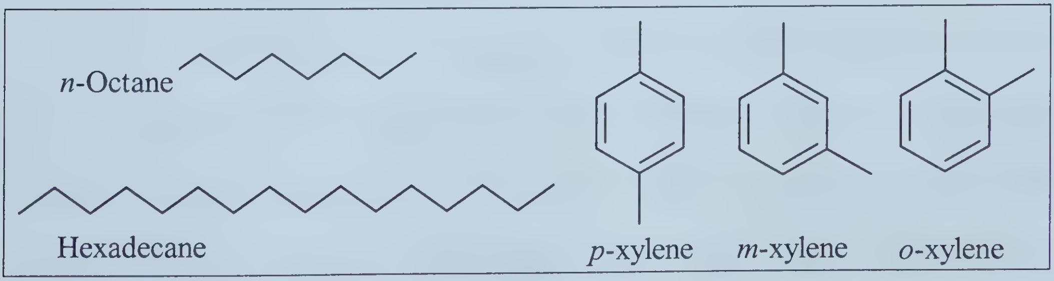


4.1.1.2 Biocatalyst Interaction with Organic Phase

As described in the section for preparing the biocatalyst, the model compound DBT was reacted in a biphasic system; therefore, it was important to understand the cell interaction with the organic hexadecane phase. The mechanism by which the biocatalyst obtains the substrate, which is dissolved in an organic phase, has a significant impact on rate of conversion.

The hydrophobicity of the LP6a mutant was tested using a method developed by Rosenberg et al. (1980b). The test examines the attachment of LP6a to an organic phase, which is the potential carrier of the substrates, in this case *n*-C_{16}. Two positive control bacterial strains, *A. calcoaceticus* RAG-1 and *A. calcoaceticus* BD 413 were used and the organic compounds were *n*-C_{16}, *n*-octane, and xylenes. These compounds, as shown in Figure 4-5, represent 2 classes of chemical structures.

Figure 4-5: Organic compounds used in the hydrophobicity test



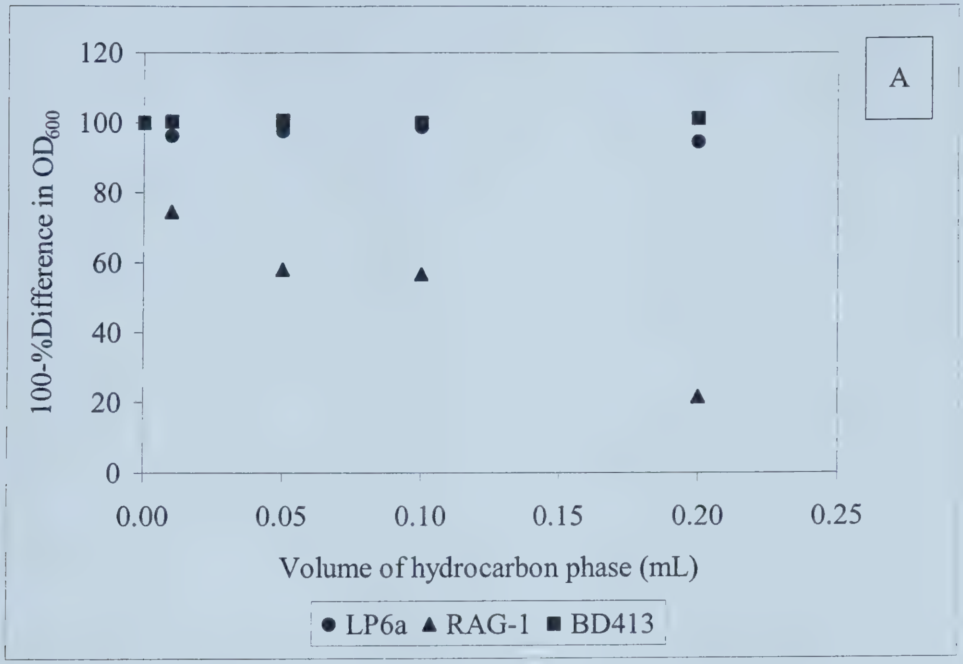
The three bacterial strains were grown as in section 4.1.1.1. The cultures were incubated for 72 hr at 28 °C on a rotary shaker at 200 rpm. From each culture, a sample

was taken after 24 hr and 72 hr. The 10 mL samples were centrifuged at 16,300x g for 10 min. The pellets were washed twice with 10 mL buffer and pelleted again. The washed pellets were resuspended in buffer to an $OD_{600} \leq 0.5$. Several 13 mmx100mm test tubes were filled with 1.2 mL each of the cell suspension. The test was performed at room temperature. The different hydrocarbons with varying volumes were added and mixed vigorously for 2 min using a vortex mixer. The phases were allowed to separate for 10 min before measuring OD_{600}. The aqueous phase OD_{600} was monitored before and after the mixing, which corresponded to the initial and final OD_{600} respectively. Figure 4-6, Figure 4-7 and Figure 4-8 present the 24 hr and 72 hr results for hexadecane, *n*-octane and xylenes, respectively. As the organic phase volume was increased from 0 to 0.2 mL, the initial OD_{600} of RAG-1 was reduced, but not LP6a or BD 413. The same results were observed for each of the three hydrocarbon compounds. The results were consistent with the literature reports for the positive control RAG-1, but not for BD 413, which was expected to follow RAG-1 (Rosenberg et al., 1980b). The loss of BD 413 ability to adhere may be due to storage. Nevertheless, the data for RAG-1 verified the effectiveness assay for the measuring cell hydrophobicity.

The results from Figure 4-6, Figure 4-7, and Figure 4-8 indicated that late and early stationary phase LP6a mutant #21-41 had no affinity for the organic phase. The initial experimental data for LP6a had a standard deviation of ± 0.017. The experimental procedure in this work depends on dissolving the different PAC (e.g. DBT) in an organic phase carrier (e.g. hexadecane). Based on these data, we suggest that the mutant utilize the dissolved PAC in the aqueous phase without adhering to the organic layer. Such a conclusion is supported in the literature, where studies showed that in two liquid phase

systems, the substrate often diffuses from the water-immiscible phase to the aqueous phase, and the microorganisms may transform the substrate at the aqueous phase (Efroymson & Alexander, 1991, Marcoux et al., 2000).

Figure 4-6: Cell hydrophobicity test, determined as optical density of the aqueous phase as function of volume of *n*-hexadecane and culture age. (A) The cultures were used after 24 hr of growth. Adherence is indicated by reduction in OD_{600} relative to initial OD_{600} (B) The cultures were used after 72 hr of growth



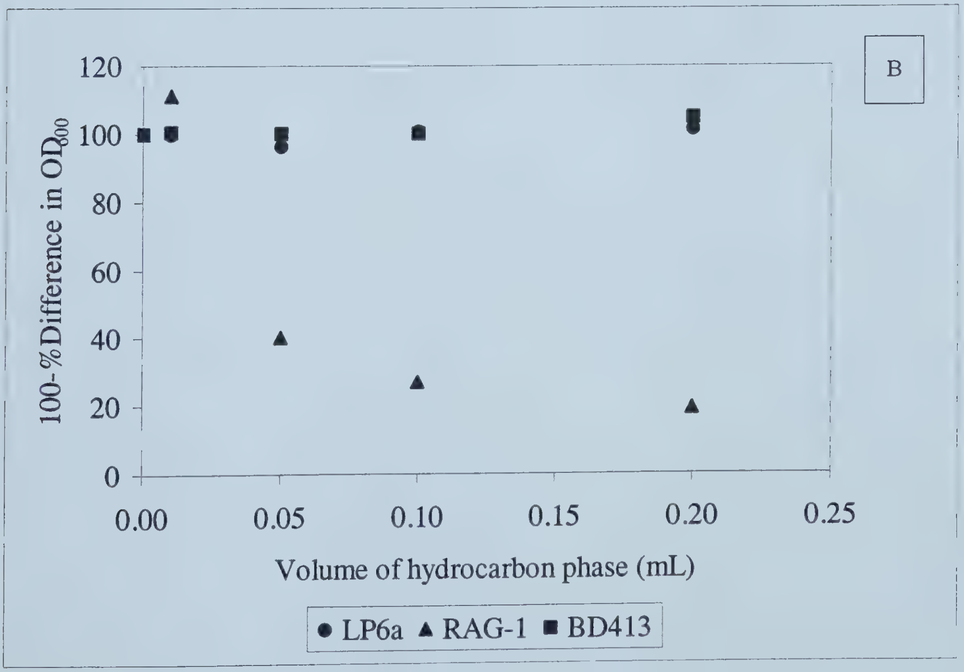
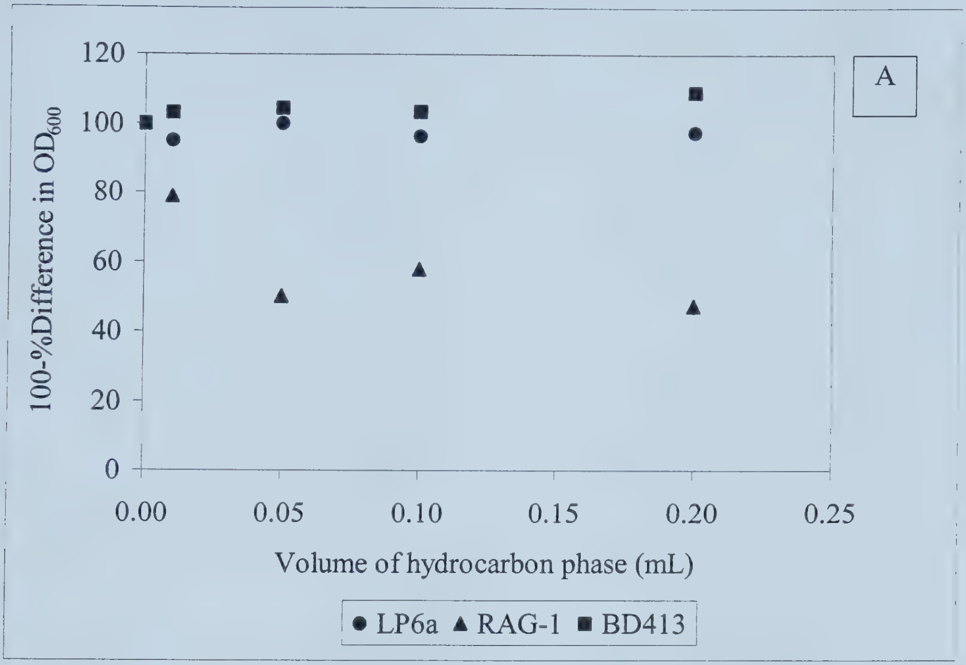


Figure 4-7: Cell hydrophobicity test, determined as optical density of the aqueous phase as function of volume of *n*-octane and culture age. (A) The cultures were used after 24 hr of growth. Adherence is indicated by reduction in OD_{600} relative to initial OD_{600} (B) The cultures were used after 72 hr of growth



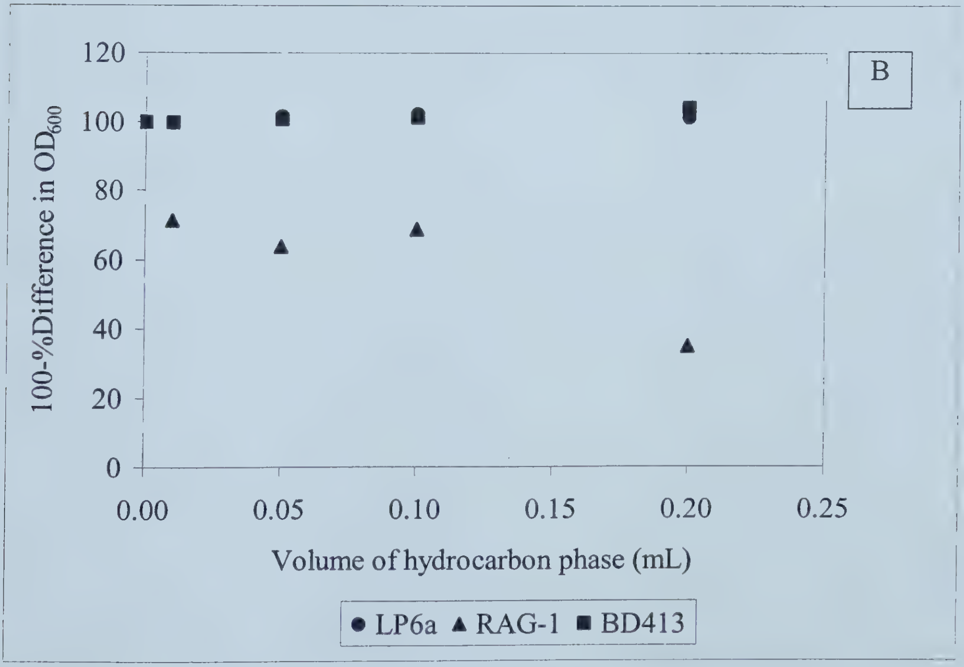
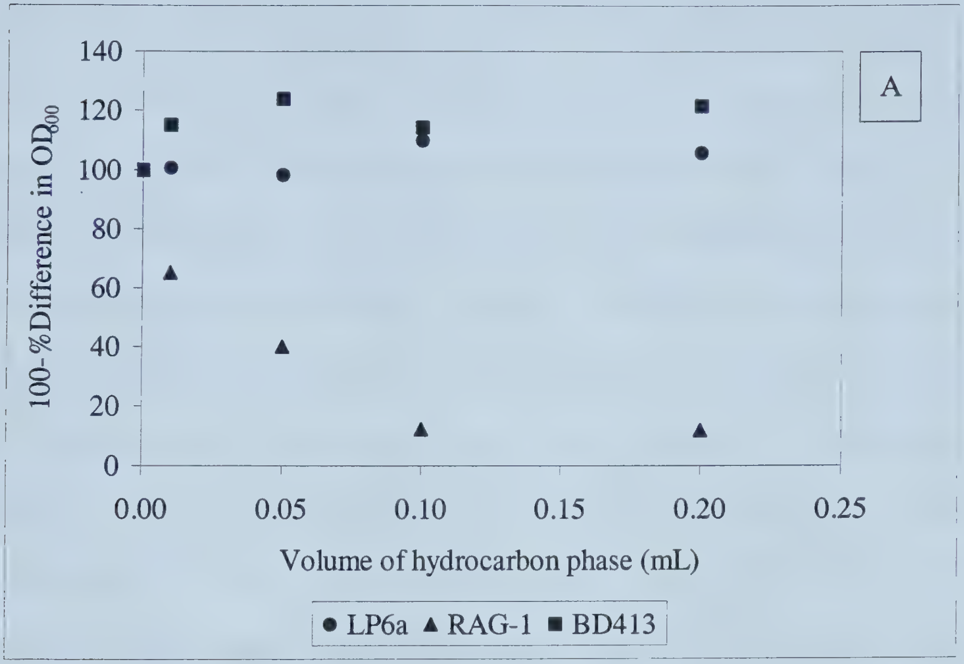
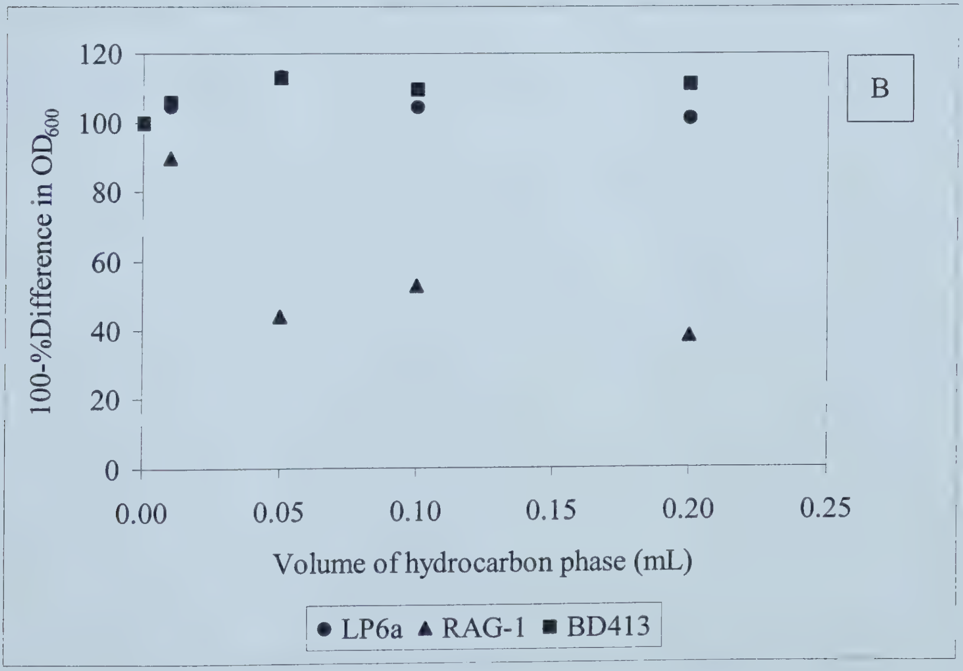


Figure 4-8: Cell hydrophobicity test, determined as optical density of the aqueous phase as function of volume of xylene and culture age. (A) The cultures were used after 24 hr of growth. Adherence is indicated by reduction in OD_{600} relative to initial OD_{600} (B) The cultures were used after 72 hr of growth





4.1.1.3 Biocatalyst Viability During Experiments

Because the LP6a biocatalyst was used in a resting state suspended in buffer, it was necessary to clarify that any observed alteration in the biocatalyst activity was not due to a reduction in the number of viable cells. Thus, the induced biocatalyst was prepared in two 500 mL batches and each was divided into 2x200 mL volume. Two 200 mL batches exposed to DBT in C_{16}, and the other two had neither DBT nor C_{16}. The two treated batches had a concentration of 1 mmole/L of DBT, and appropriate amount of 2AB. The batches were shaken for three cycles; each cycle was 24 hr, at 28 °C and 200 rpm. After each cycle the biocatalyst pellets were collected with centrifugation at 16,300xg for 10 min. The pellets were washed with 100 mL buffer, which was removed by centrifugation. Then they were resuspended each in 200 mL buffer, where DBT was introduced to the assigned batches again as well as 2AB. The experiment measured OD_{600}, viable count and the total protein content. The results for changes in OD_{600} are shown in Figure 4-9, for viable count in Figure 4-10, and for total protein content in Figure 4-11.

Figure 4-9: OD_{600} measurement during a three-cycle experiment. Each cycle was 24 hr. Batches A and B were not exposed to DBT, meanwhile Batches C and D were exposed to DBT in hexadecane

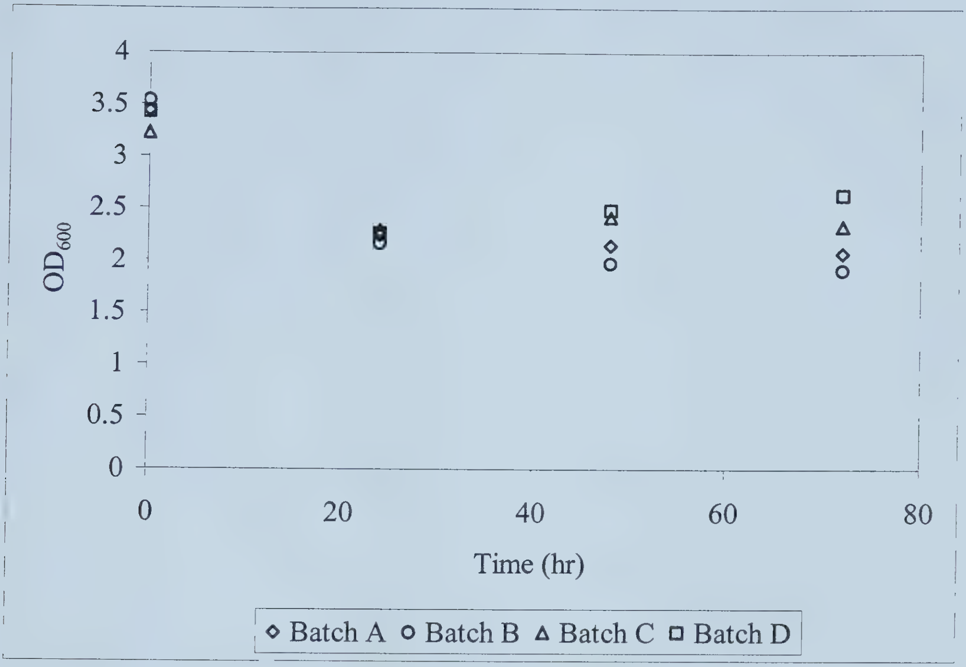


Figure 4-10: Viable count measurements during a three-cycle experiment. Each cycle was 24 hr. Batches A and B were not exposed to DBT, meanwhile Batches C and D were exposed to DBT in hexadecane

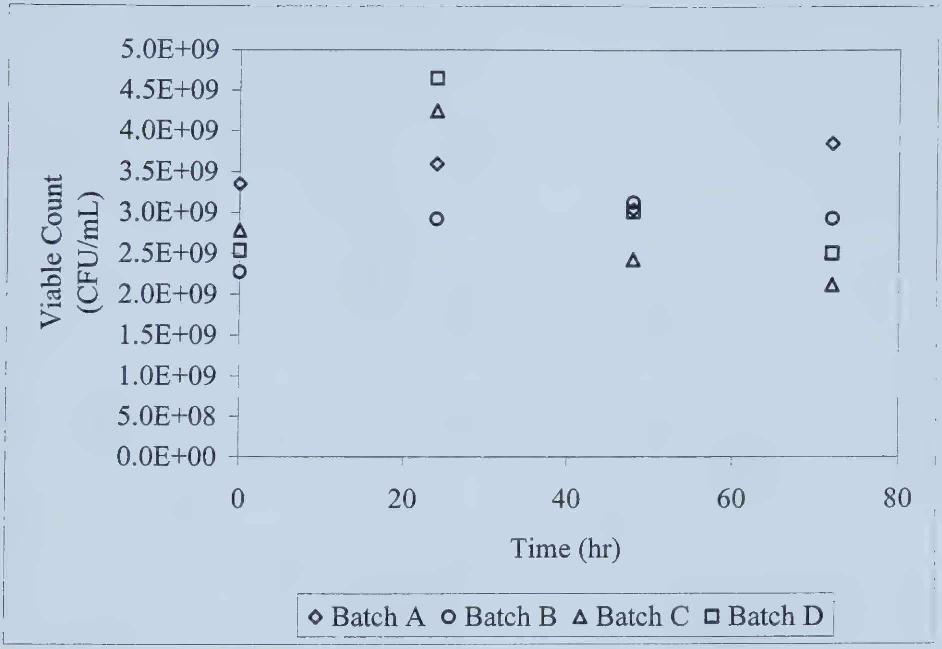
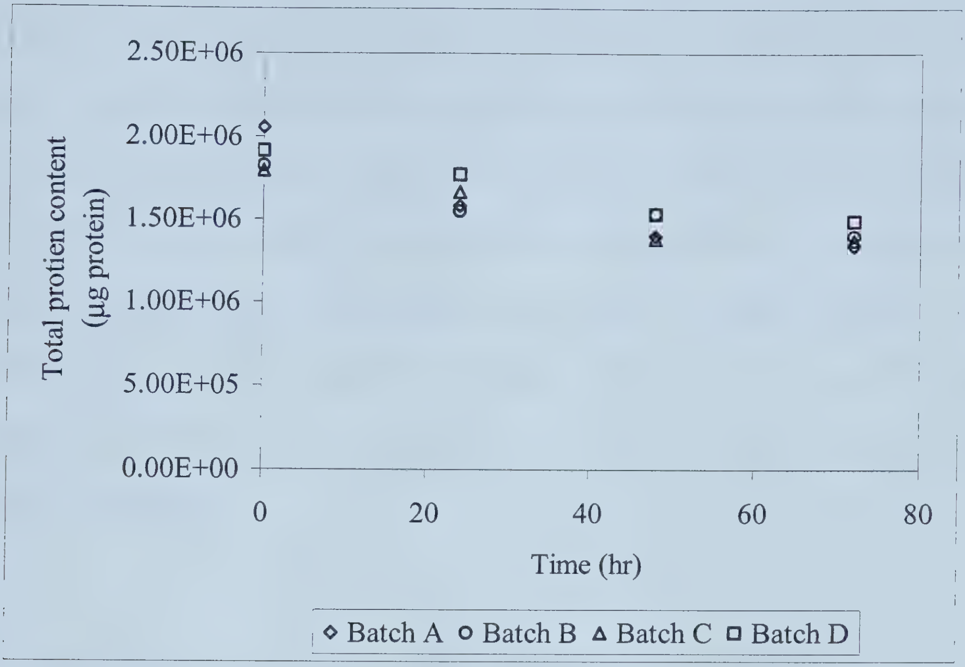


Figure 4-11: Protein content measurements during a three-cycle experiment. Each cycle was 24 hr. Batches A and B were not exposed to DBT, meanwhile Batches C and D were exposed to DBT in hexadecane



One observation was that viable cell numbers increased slightly after the initial samples. This could be due to division of cells that were ready to divide while in growth medium, or separation of clumped cells. However the latter reason was unlikely since the resuspended cells were separate when examined under the microscope. Although there were slight variations between the exposed and unexposed batches, the viable count throughout the experimental time was stable on average. The other two measurements were changing, more likely due to centrifugation. From this we conclude that any alteration in biocatalytic activity for experiments up to 72 hr duration was not related to reduction in viability.

4.1.2 Efficiency of Extraction and DBT Evaporation Rate

4.1.2.1 Recovery of Organic Carrier Phase

Since the organic phase was recovered by liquid-liquid extraction, it was necessary to determine the extraction efficiency. Two batches of 200 mL biocatalyst were prepared and 2 mL of pure n-C_{16} was added. The two batches were incubated at 28 °C and shaken for 24 hr at 200 rpm, after which pentane extraction was done. Four empty vials were weighed: 2 mL of fresh n-C_{16} was added separately to two vials, and the extracted n-C_{16} was added to the other two vials. The former vials were used as references of the accuracy of pipetting and the other two vials were the experimental vials. The difference in weight between the empty and full vials represented the weight of n-C_{16} ($W.C_{16}$). Then, the n-C_{16} volume ($V.C_{16}$) was calculated by the multiplication of

($W.C_{16}$) and n-C_{16} density reciprocal ($1/\rho C_{16}$). The results in Table 4-1 showed a 98.9 percent recovery of the 2 mL n-C_{16}.

Table 4-1: Extraction efficiency (averaged results)

Added volume of n-C_{16} (mL)	**Added n-C_{16} by weight (g)**	**Averaged volume recovered (mL)**	**Averaged recovered weight (g)**
2.00	1.55	1.98	1.54

4.1.2.2 Loss of DBT by Evaporation

Kropp et al. (1997) showed a 12% loss of DBT by evaporation during 7 days of incubation. Therefore, a test was conducted to find the evaporation rate of DBT within a 24 hr experiment. Four 200 mL abiotic batches were prepared with only buffer and 2 mL DBT solution (0.1 M). Two batches were extracted with pentane at time zero, while the other two were shaken for 24 hr at 28 °C and 200 rpm, then they were extracted. The recovered DBT was analyzed using the GC-FID. Including the 98.9% extraction efficiency, the DBT loss from the initial batches was less than 1 µmole but without considering the extraction efficiency it became 0.5% (1 µmol). The results of the 24 hr batches with and without considering extraction efficiency were 1.6% (3 µmole) and the 2.6% (5 µmole), respectively. The average losses of DBT were minor compared to the amount added (190 µmole). In comparison, the 12% loss observed by Kropp et al. (1997) was from an initial amount of 21 µmol per flask (i.e. the loss was 2.5 µmol).

4.1.2.3 Repeatability and Error analysis

The measurements mostly were based either on the spectrophotometry, or GC. The spectrophotometer was used for measuring cell density at 600 nm, the protein content in samples and DBT absorbance in the ultraviolet (UV) wavelength range. The GC instrument was used to measure the remaining (unconverted) DBT in the biocatalyst. The error analysis is presented as the average value ± the standard deviation.

To evaluate the accuracy of dividing a biocatalyst from a large volume batch into batches with smaller volume, a 2.5 L biocatalyst was prepared and divided into 12x200 mL batches, where 100 μL sample was taken and diluted 10 times. The average OD_{600} equaled 3.54 ± 0.14. To evaluate the accuracy of measuring OD_{600} from a single batch, a 3x100 μL volume were taken and diluted 10 times in ddH_2O. The average OD_{600} was 3.57 ± 0.15. Therefore, the spectrophotometer measurements for cell density (OD_{600}) were accurate to better than 5% error.

The accuracy of measuring the protein content of a batch of cells was tested. A large biocatalytic volume was divided into 3x200 mL batches, where a 1 mL sample was taken from each batch and analyzed twice. The average protein content was $7.15 \pm 0.74 \times 10^3$ μg protein/mL for a 200 mL culture. Since the measuring procedure is standard and based on a standard protein kit, then it was possible to consider the value 0.74×10^3 μg/mL protein as the error in measuring the protein content also in a 30 mL culture.

The precision in measuring the remaining DBT in a flask was tested for 4 batches, each with 30 mL biocatalytic volume. Two batches had initially 28.5 μmole DBT (i.e. 1 mmole/L) the other two had 71.4 μmole of DBT (i.e. 2.5 mmole/L). The batches were originally from a batch with a larger volume. The samples were terminated at time zero,

by adding 200 μL (6 N) HCl then they were neutralized by adding 120 μL (10 N) NaOH. The remaining DBT was extracted with 4x20 mL pentane. For the 1 mmole/L batch and the 2.5 mmole/L batch, the GC data showed a an average loss of 4.43 ± 1.07 μmole DBT, and 7.71 ± 2.45 μmole DBT, respectively. Since the samples were collected at time zero, it was assumed that no reaction had taken place. These losses indicated the cumulative error due to evaporation, and extraction in the presence of cells.

The DBT converted was calculated as the moles of DBT converted per mass of total protein. By using equation (4-1) and from the previous results for the two units, we estimated the accuracy in calculating the converted DBT.

$$\partial z = \left|\frac{X}{Y}\right|\sqrt{\left(\frac{\partial x}{X}\right)^2 + \left(\frac{\partial y}{Y}\right)^2} \qquad \textbf{(4-1)}$$

Where,

∂z = The standard deviation of Z the average converted DBT in units (μmole DBT/μg protein)
∂x = The standard deviation of X the average converted DBT (μmole DBT)
∂y = The standard deviation of Y the average total protein content (μg protein/mL)

The values of converted DBT had an error of (± $1.6\text{x}10^{-4}$ μmole DBT/μg protein/mL culture) for a culture with an initial concentration of 1 mmole/L. The values of converted DBT had an error of (± $3.6\text{x}10^{-4}$ μmole DBT/μg protein/mL culture), for a culture with an initial concentration of 2.5 mmole/L.

4.1.2.4 Correlation between OD_{600} and Total Protein Content

OD_{600}, dry weight and the total protein content are alternate measures of the total biomass present in a biocatalyst suspension. The dry weight measurements were not used

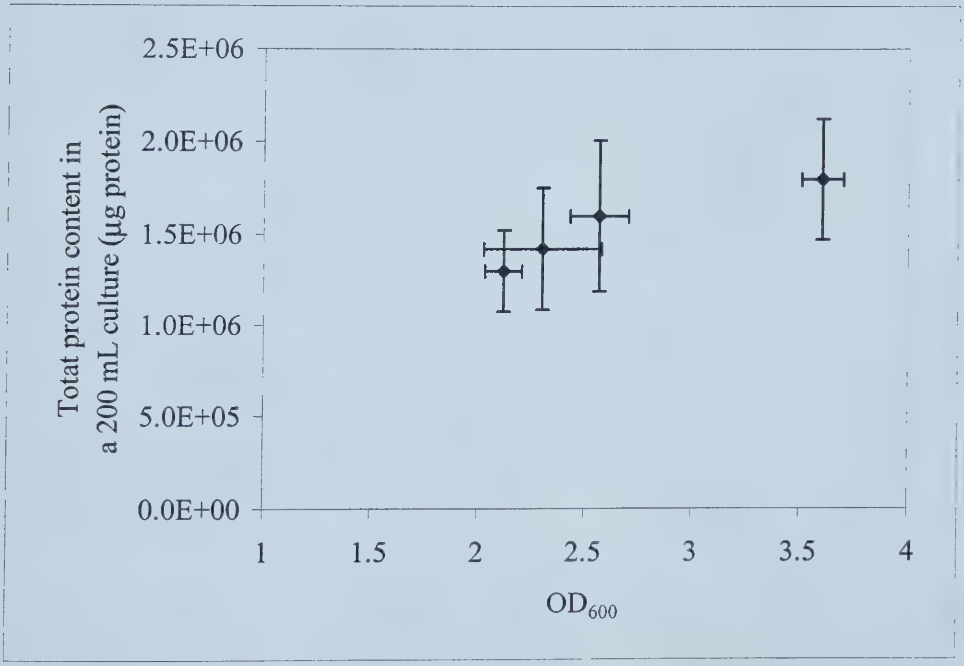
frequently due to the long time needed to dry the samples. The two preferred measurements were OD_{600} and total protein content. The procedure for both measurements was described in the previous chapter. In order to correlate between the two measurements, three batches of suspended cells were analyzed. The batches were generated from 3 independent large volumes of biocatalyst. The batches each were of 200 mL, and they were incubated at 28°C for 24 hr and shaken at 200 rpm. Then the batches were centrifuged independently for 10 min at a speed of 16,300xg. The collected pellets were resuspended in 100 mL buffer and centrifuged for 10 min at 16,300xg. The collected pellets were resuspended in 200 mL buffer, and the process was repeated three times (i.e. over a period of 72 hr). Triplicate samples were taken for the two measurements at the beginning of each time interval. The repeated process of incubation, washing and centrifugation was designed to mimic the preparation of the cells and to determine the ratio between the OD_{600} and protein content with time.

The data in Table 4-2 present the average values of three batches, for the two measurements at the beginning of each time interval. The reduction in both measurements showed consistency. However, as shown in Figure 4-12, by drawing the error bars which were the standard deviations, the values statistically were not different and the linear correlation was poor. Therefore it is recommended that protein content and OD_{600} be measured for every experiment, and that was the procedure followed for all the experiments conducted in this work.

Table 4-2: Reduction in OD_{600} measurement and total protein content for 4 consecutive runs. Each experiment was for 24 hr, and values were taken at initial time

Time (hr)	OD_{600}	Total protein content (µg protein)	OD_{600}/Protein content
0	3.6	1.8 E 6	2.0 E 6
24	2.6	1.6 E 6	1.6 E 6
48	2.3	1.4 E 6	1.6 E 6
72	2.1	1.3 E 6	1.6 E 6

Figure 4-12: Correlation between OD_{600} and total protein content



4.2 Kinetics of DBT Conversion and Metabolite Formation

DBT was used as the model compound to determine rates of ring opening because: (1) it is less volatile than naphthalene and (2) its water-soluble ring cleavage products have an absorption maximum at 475 nm that was readily quantified spectrophotometrically. Therefore we attempted to correlate the color intensity with the conversion of DBT. The finding of such a correlation would permit rapid quantification of ring cleavage without solvent extraction and GC analysis. According to the Beer-Lambert Law, shown in Equation (4-2), there exists a linear relationship between absorbance of a solute and the concentration of this solute provided the path length through the cell containing the solute is constant.

$$A = \varepsilon c L \qquad \textbf{(4-2)}$$

Where,

A = absorbance

ε = extinction coefficient of solute

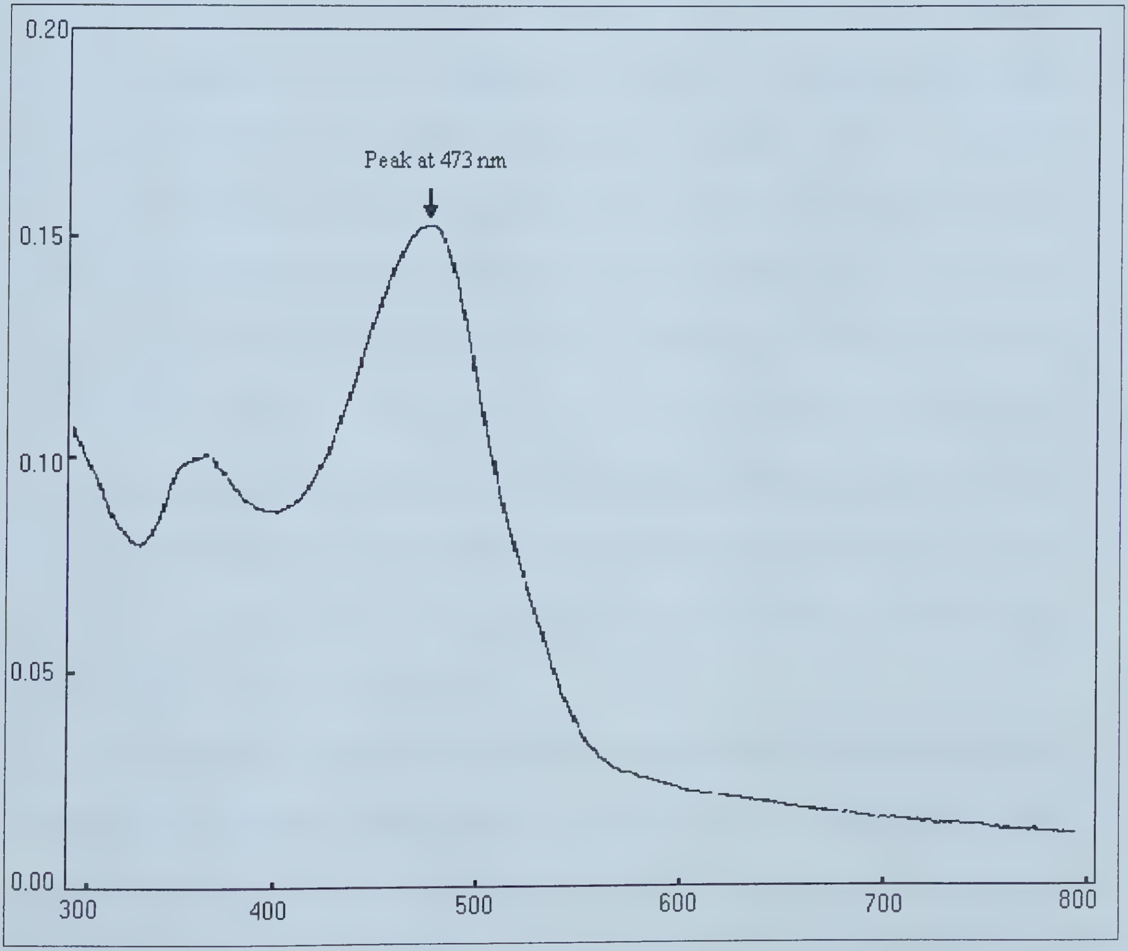
c = concentration of solute

L = length of light path

The solute was the open ring product. In the following experiments the spectrophotometer sample cells had a 1-cm path length. The main assumption was that DBT was converted to a single open ring product, and the absorbance at 475nm corresponded solely to this product. This assumption was tested, by analyzing a sample taken from a 24 hr used biocatalyst. The aqueous phase of the biocatalyst had an

absorbance peak at 473 nm, as shown in Figure 4-13, the slight variations due to instrument lack of calibration. Thus the relation in the concentrations between the converted DBT to the open ring product was assumed to be 1 to 1, and the solute concentration corresponded to the amount of DBT converted. The objective therefore was to determine the extinction coefficient, ε in equation (4-2).

Figure 4-13 Absorbance of the biocatalyst aqueous phase for a 24 hr experiment



4.2.1 Metabolite Absorbance During 28 hr Experiment

The previous section suggested that the metabolite have an absorption maximum at 475 nm. Based on the detailed experimental method given in section 3.5.1, the data in Figure 4-14 present changes in rate of formation of metabolites for a 28 hr run. The rate was calculated from the slopes as change in absorbance unit per min (AU/min). The linear relation between time and absorbance changed from 0.003 AU/min in the initial 200 min to 0.013 AU/min from 4th hr to 20th hr, and to 0.006 AU/min in the remaining 6 hrs. The changes in slope for measurements at the same wavelength imply either involvement of different intermediate metabolites, which leads to the final open ring product, or the formation of some side products. Thus, we required a detailed analysis of the metabolites formed during the experimental time. If coupling is to be found, then it would not be possible to calculate the extinction coefficient (ε), since the extinction coefficient (ε) correlates the absorbance of a pure solute and its concentration in solution. The conventional method in measuring the initial enzyme activity depends on the linear relation between the metabolite absorbance and the initial 60-min. This method could be used, but with caution, since some of the metabolites are sensitive to chemical and physical changes, such as pH, and time.

The standard procedures for sampling and quantitative GC-FID analysis lead to consistent and repeatable results, within the experimental error and batch to batch variability. The data for DBT conversion were collected by sacrificing sample volumes of 200 mL and 30 mL. Figure 4-15 present the amount of DBT converted with time. Some of the data points were an average of duplicate samples. The DBT conversion was

steadily increasing with incubation time. The results did not show a leveling off of the conversion during the 28 hr experiment. Thus, a similar experiment later was conducted with an extended experimental time. The study of DBT rate of transformation was presented in Figure 4-16, where the rate of transformation was constant after the 2nd hr. The initial 3 data points were eliminated because the experimental error at time zero is reported as conversion.

The data in Figure 4-17 present an attempt to correlate the amount of DBT converted with the absorbance of metabolites at 475 nm. The linear regression was expected to have a slope of 1, but this was not the observed result. The changes in rate of absorbance in Figure 4-14 and the sigmoidal relationship in Figure 4-17 suggests that the metabolites were undergoing side reactions, giving changes in color intensity. This lack of a linear relationship; implies that the absorbance of metabolites can only be used as a qualitative measurement rather than a quantitative measure of conversion.

Figure 4-14: Changes in rate of formation of metabolites for a 28 hr run. Initial DBT concentration was 1 mmole/L

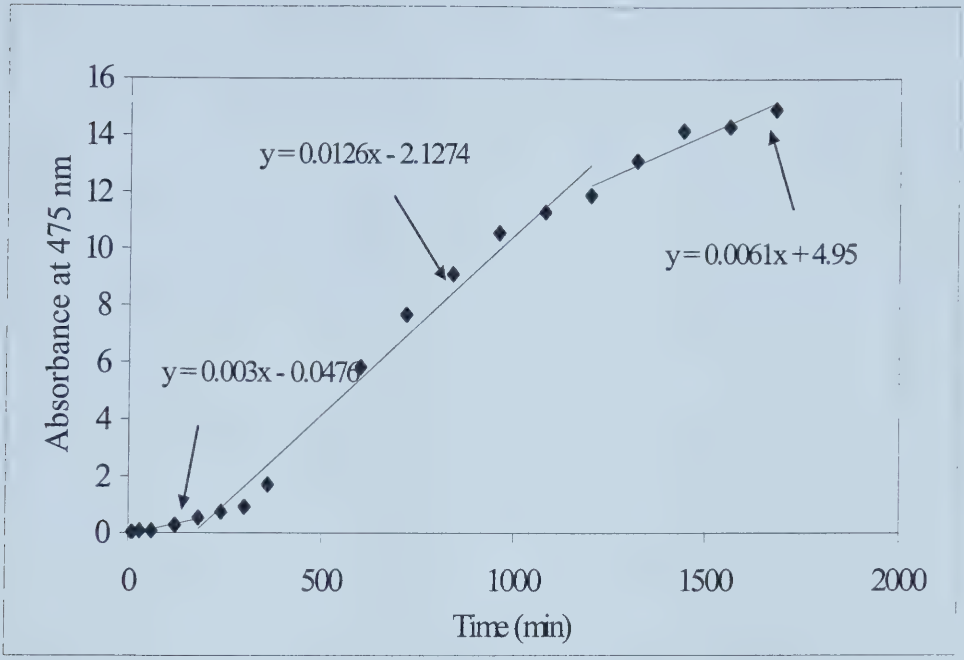


Figure 4-15: Conversion of DBT with time based on biocatalyst protein content (µmole/(µg protein). Initial DBT concentration was 1 mmole/L

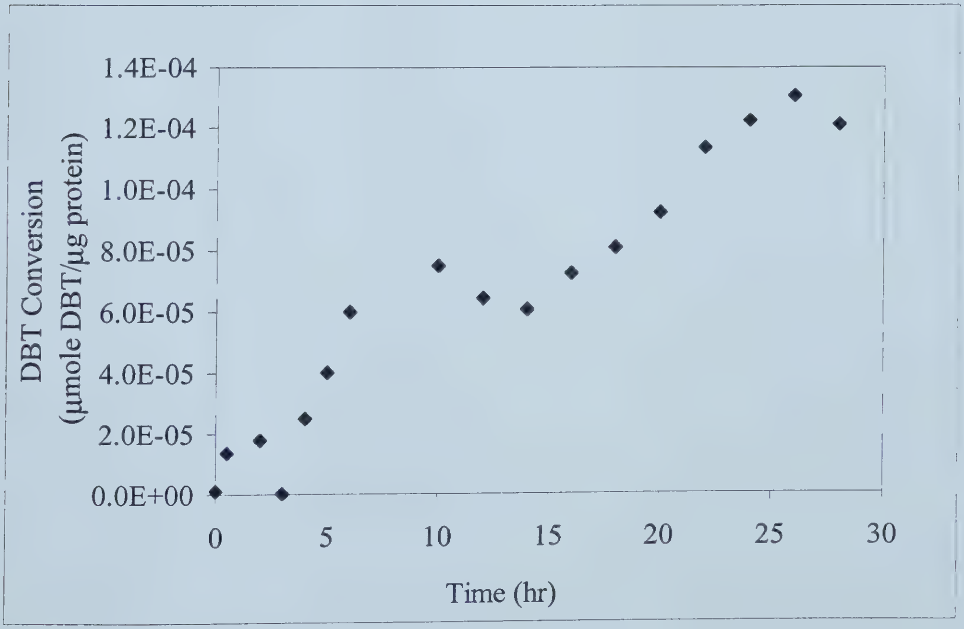


Figure 4-16: Rate of DBT conversion based on total protein content (μmole/μg protein/hr). Initial DBT concentration was 1 mmole/L

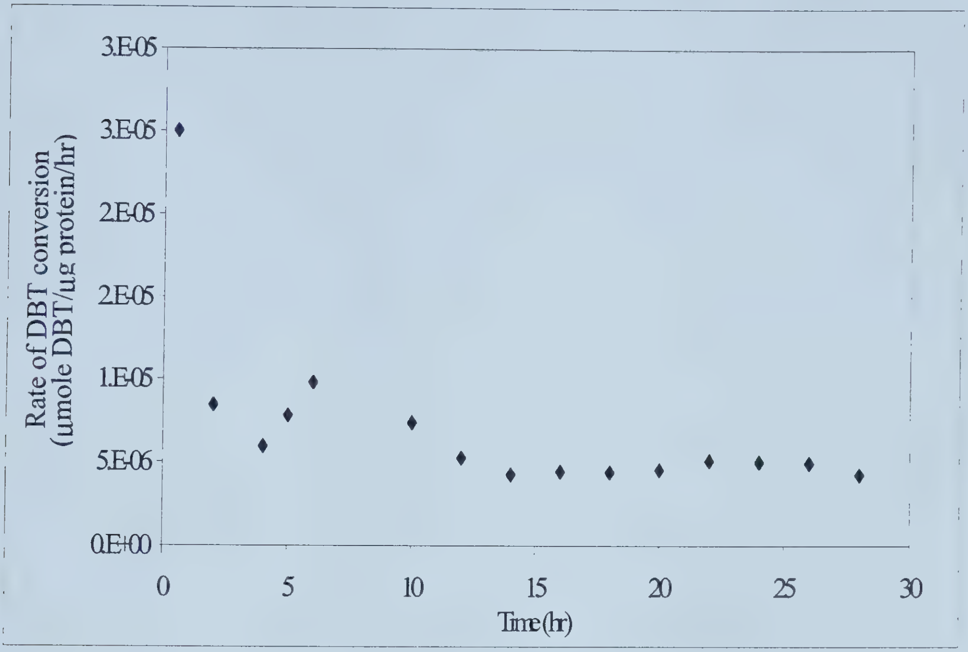
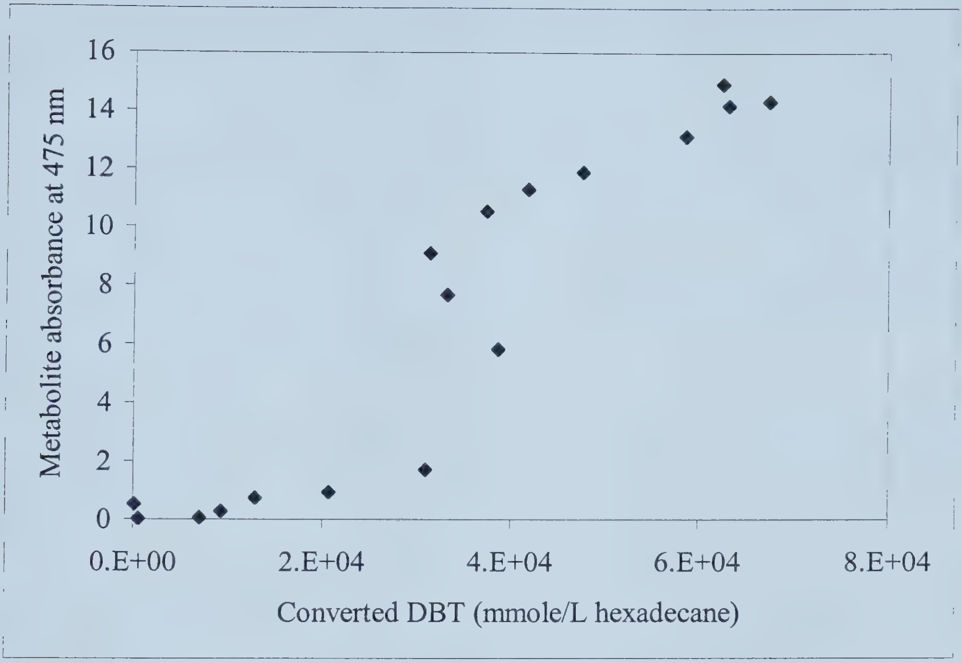


Figure 4-17: Conversion of DBT (μmole/L of organic phase) versus metabolite absorbance at 475 nm. Initial DBT concentration was 1 mmole/L



4.2.2 Analysis of DBT Metabolites from LP6a Mutant 21-41

The open ring products of DBT were polar compounds that could not be directly detected by GC-FID or extracted using the same procedure as DBT. These products required derivatization to be identified. Thus the metabolites were extracted, derivatized with diazomethane and then analyzed using gas chromatography mass spectrometry (GC-MS). Details of the method are given in section 3.4. This analysis was required to clarify the reasons for changes in slopes in Figure 4-14.

The total ion chromatographs for the different pH samples showed no significant variations in peaks, as most of the metabolites were extracted at pH = 2, data are not shown. The total ion chromatographs of the 4 hr batch and the 16 hr batch were different, as shown in Figure 4-18 and Figure 4-19. Peaks that dominated in samples from the 4 hr sample were reduced or increased or sometimes disappeared in the samples from the 16 hr samples. Some new peaks appeared in the 16th hr samples.

Figure 4-18 Total ion chromatographs of the 4 hr sample

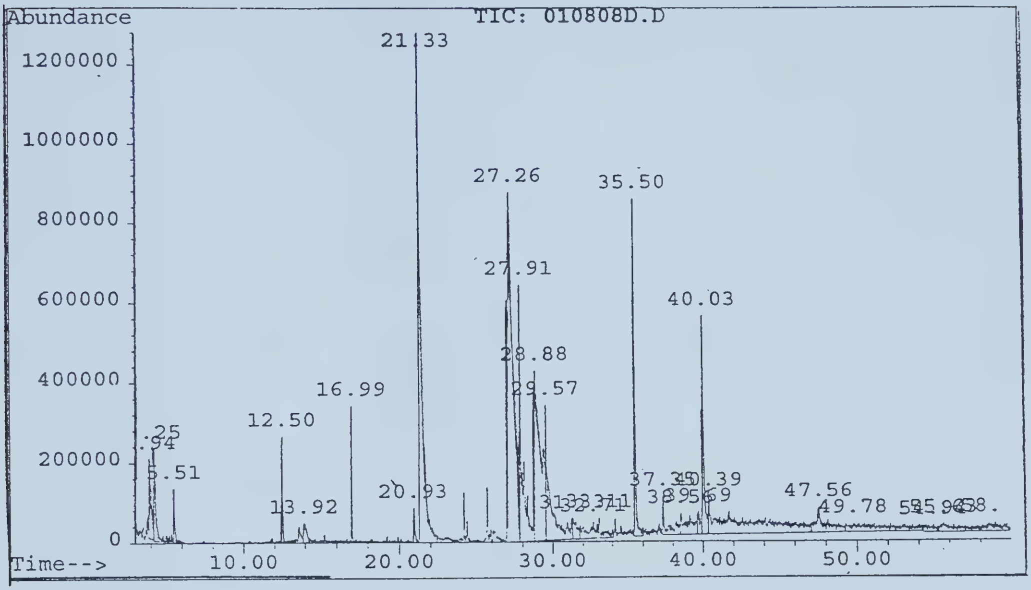
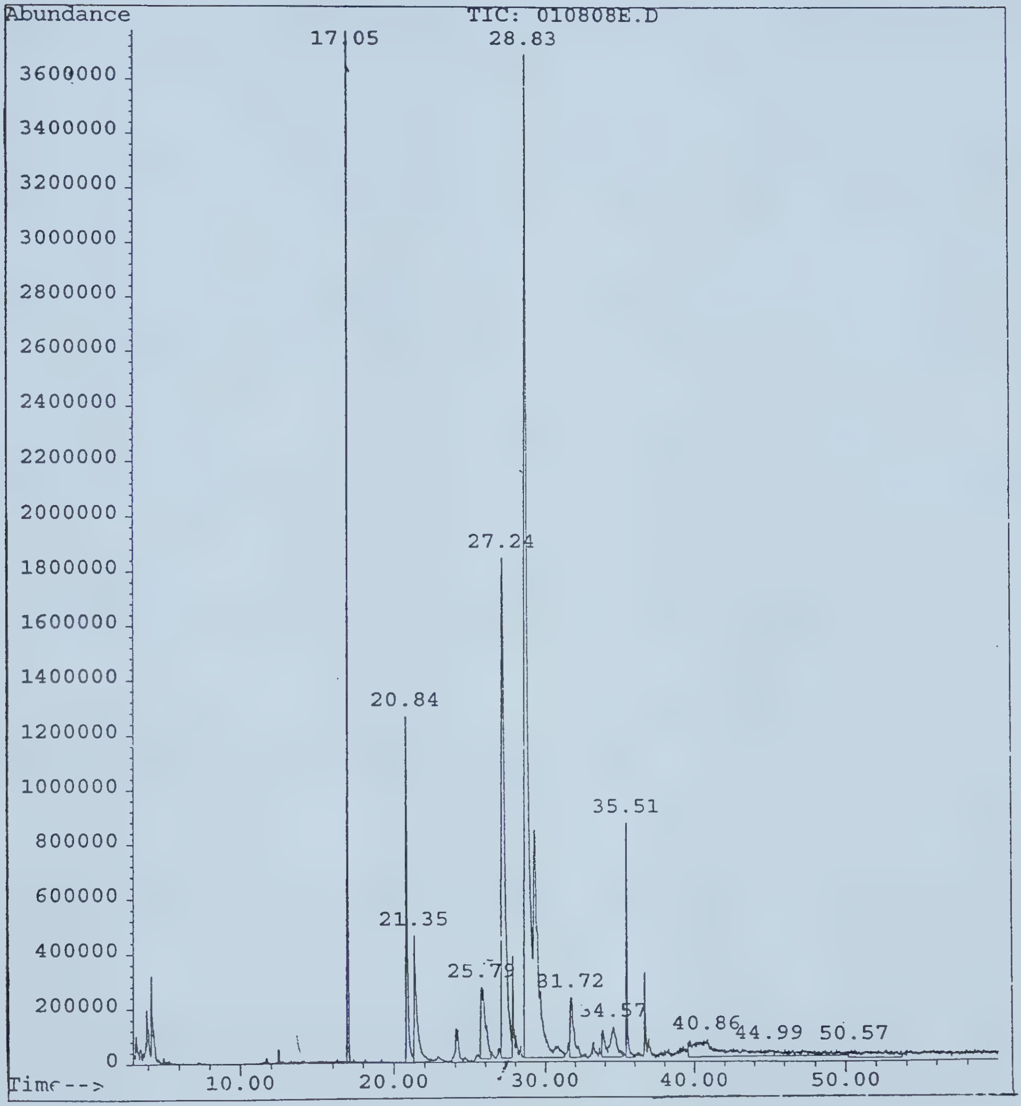


Figure 4-19 Total ion chromatographs of the 16 hr sample



The mass spectrum of the main metabolite gave comparable molecular fragments to the expected end product, as shown in structure (I) in Figure 2-11. The main difficulties in interpreting the data were that the peaks were not sharply separated, and there was evidence of incomplete derivatization where both the parent compound and its derivatized form appeared. For example, as shown in Figure 4-20, one of the end product structures had a molecular weight of 250 and when derivatized it had a molecular weight of 264. Both peaks were found in the total ion chromatogram. The fragmentation of products with molecular weight 250 and 264 are shown in Figure 4-21 and Figure 4-22, respectively. Meanwhile other possible structures were completely derivatized. For example, the end product structure with a molecular weight of 248 was derivatized and only the derivatized form appeared with a molecular weight of 262, as shown in Figure 4-20. Although the metabolite with molecular weight of 264 did not appear in the Kodama et al. (1973) pathway, the molecular fragments in general indicated that the mutant follows the pathway for biotransformation of DBT.

The biocatalyst after a long period of incubation had varying ratios between the chemical structures of the final open ring product. This variation in ratios explains the gradual changes in slope in Figure 4-14, since each compound has a different molar extinction coefficient. Nevertheless, for short time experiments these variations were minor, and thus metabolite absorbance showed a constant rate of formation, as shown in Figure 4-14, for the first 2 to 4 hrs. Thus, for short time experiment the metabolite absorbance at 475 nm is a good indicator of DBT conversion.

Figure 4-20: Mass spectrum of the presumed metabolite

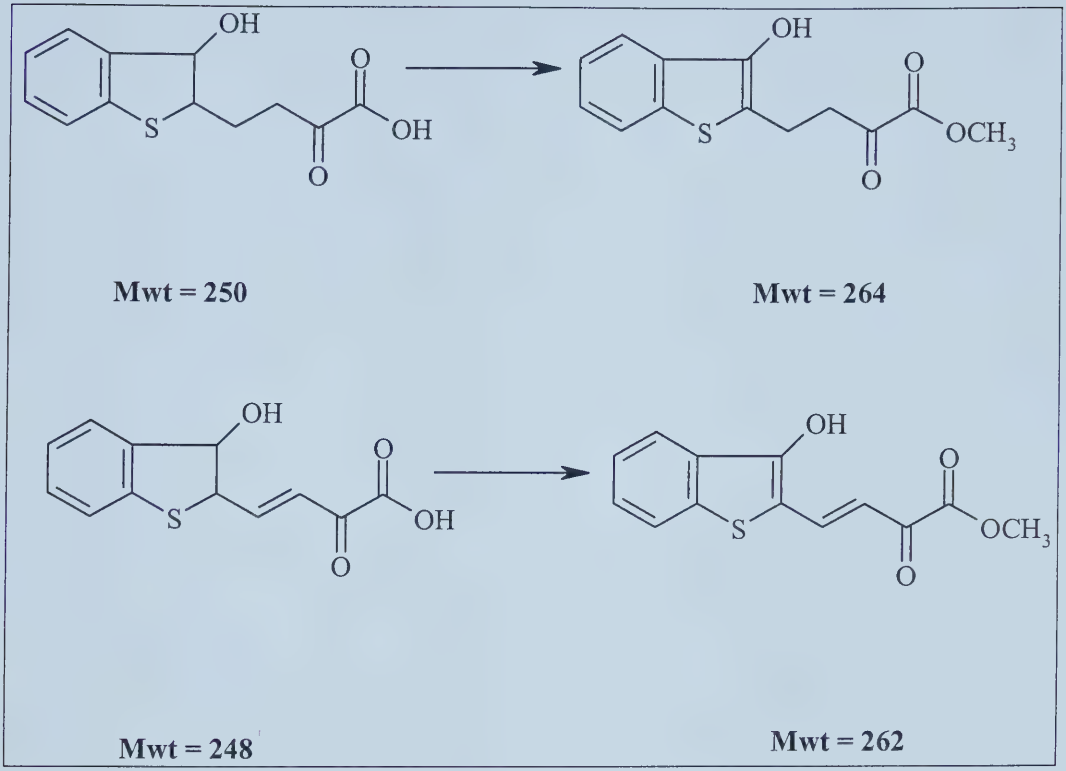

OH
O
OH
S
O
Mwt = 250
OH
O
OCH3
S
O
Mwt = 264
OH
O
OH
S
O
Mwt = 248
OH
O
OCH3
S
O
Mwt = 262

Figure 4-21: Mass spectrum of the metabolite with a molecular weight of 250

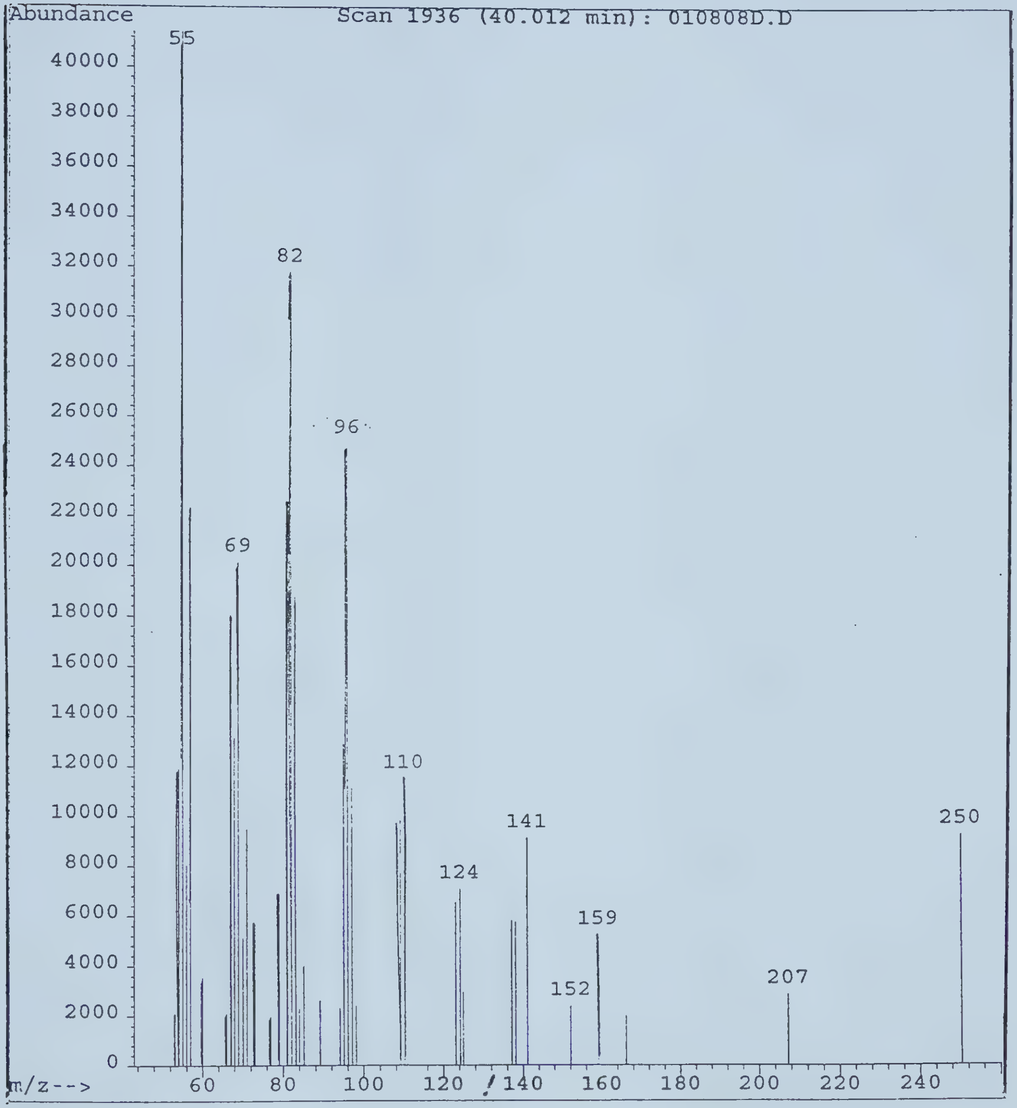
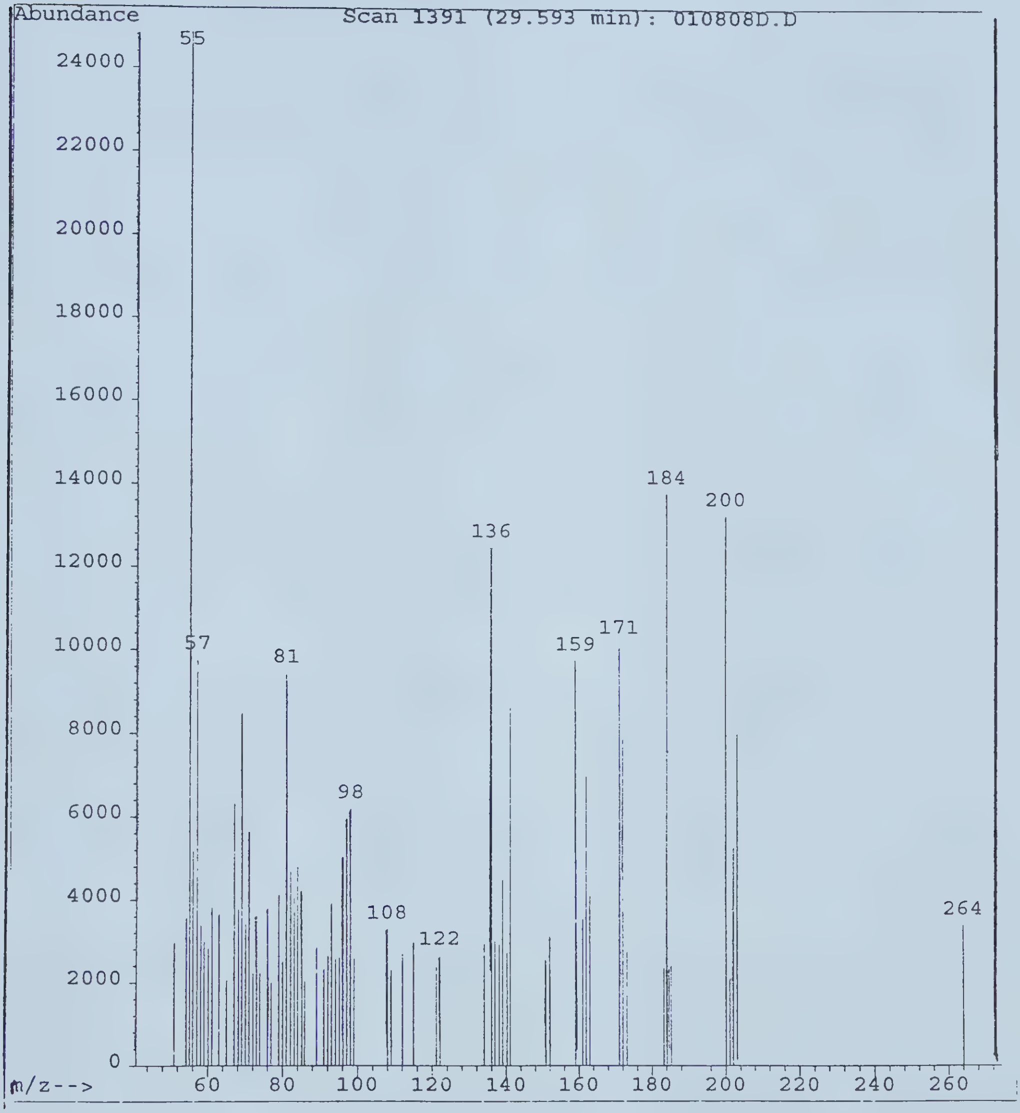


Figure 4-22: Mass spectrum of the metabolite with a molecular weight of 264

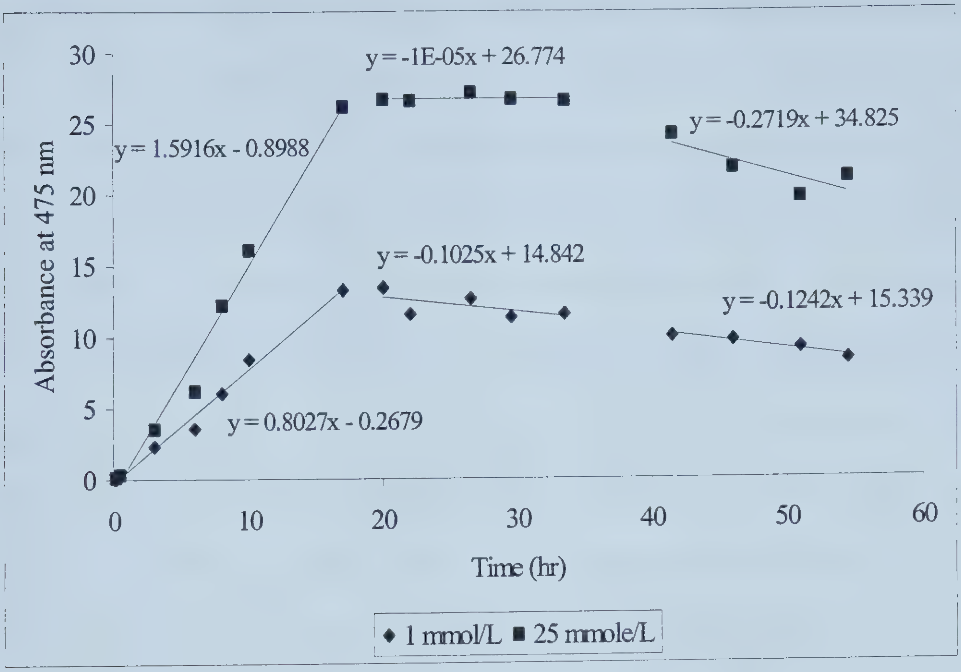


4.2.3 DBT Conversion During a 51 hr Experiment Starting with Two Different Initial Concentrations

After studying the biocatalyst activity with a single concentration of DBT, and identifying some of the metabolites, it was necessary to examine different concentrations in order to study the reaction kinetics. Variation in the concentration of substrate will resolve the reaction limiting or controlling factors, within the experimental conditions, i.e. incubation temperature at 28 °C, and mixing at 200 rpm. The ratio of organic carrier to the aqueous phase was 1:100, thus the constant mixing speed and constant organic ratio would presumably keep the organic droplet size constant. Thus, the only variable in these tests will be the initial concentration of DBT in hexadecane. Details of the experimental protocol are given in section 3.5.2.

The absorbance data of the metabolites for both concentrations are shown in Figure 4-23. The absorbance curve increased to 12 and 27 absorbance units for concentrations (1 mmole/L) and (2.5 mmole/L), respectively. The initial rate of formation for the (2.5 mmole/L) batch was double the (1 mmole/L) batch. The curves leveled off from the 18th hr to 35th hr for both concentrations, after which they declined slowly. The results of this trial were consistent with the data of section 4.2.1. The initial incremented slope implied metabolite formation. The plateau suggested that the system was saturated with metabolites. The decline after 35 hr may be due to formation of condensed metabolites that had a different maximum absorbance. In addition, a colored precipitate was observed in some experiments, which is consistent with loss of metabolites from solution.

Figure 4-23: Metabolite absorbance at 475 nm for initial concentration 1 mmole/L and 2.5 mmole/L from a 200 mL biocatalyst volume

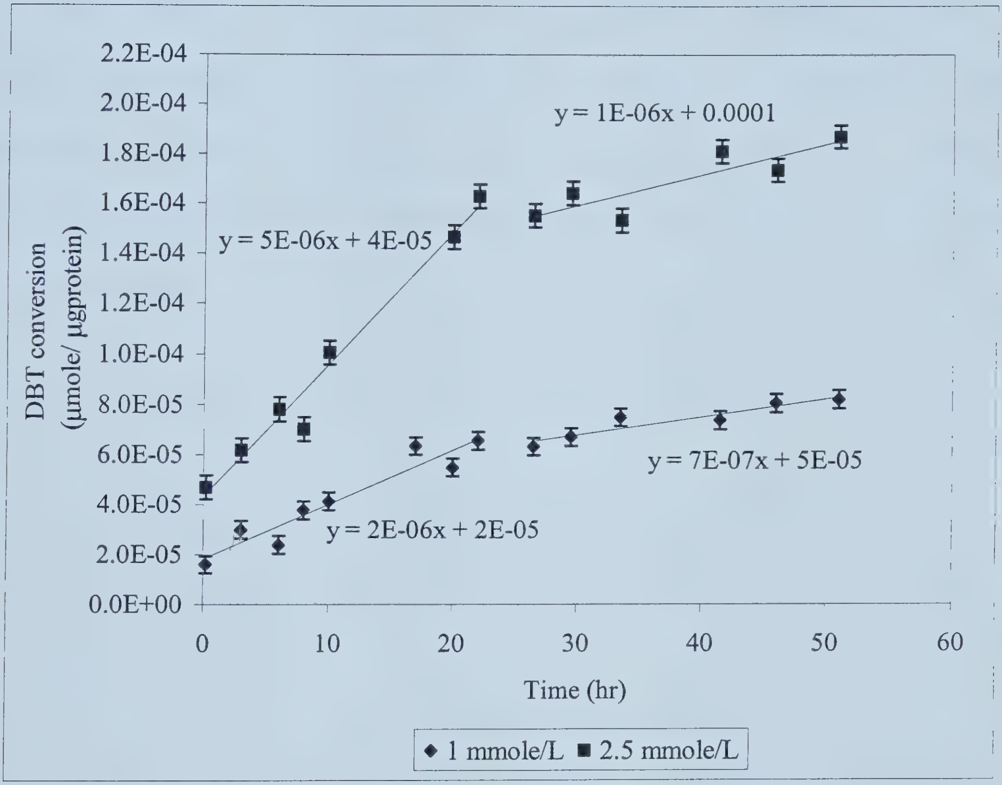


The data in Figure 4-24 show the average amount of DBT converted, normalized to the biocatalyst total protein content. Each point was the average of duplicate samples. The error bars are estimates, calculated from the average of the differences between duplicate samples. For batches with 1 mmole/L concentration the error bar was 3.44×10^{-6} (μmole/μg protein), and for batches with concentration of 2.5 mmole/L the average error bar value was 4.7×10^{-6} (μmole/μg protein). For batches with 1 mmole/L concentration the average conversion after 51 hr was 8.2×10^{-5} (μmole/μg protein), and for batches with concentration of 2.5 mmole/L the average conversion was 1.87×10^{-4} (μmole/μg protein). Consequently, the conversion of DBT was approximately linear with initial concentration. In addition, 1 mmole/L DBT gave conversions of approximately 70% after 22 hr and 90% after 51 hr. For the 2.5 mmole/L batches the average conversion was 70% after 22 hr and 83% after 51hr.

The rate of transformation of DBT changed with time, and varied between the two concentrations, as illustrated in Figure 4-24. For the 1 mmole/L concentration the initial rate for the 22 hr was 2×10^{-6} (μmole/μg protein/hr) followed by a rate of 7×10^{-7} (μmole/μg protein/hr) for the remaining 24.5 hr. For the 2.5 mmole/L concentration the initial rate for the 22 hr was 5×10^{-6} (μmole/μg protein/hr) followed by a rate of 1×10^{-6} (μmole/μg protein/hr) for the remaining 24.5 hr. Reductions in the rate of transformation could be a result of loss of the biocatalytic activity or metabolite inhibition or limitation of substrate available. The apparent difference in rate of DBT transformation between the two initial concentrations was likely due to higher aqueous concentrations of DBT. In section 4.2.4.1 we will explain the effect of the organic phase concentration on the

aqueous concentration. Meanwhile it was necessary to repeat the previous experiment to ensure the repeatability of the observation.

Figure 4-24: Conversion of DBT with time based on biocatalyst total protein content over 51 hr. The data points are means of replicate flasks



4.2.4 DBT Conversion During a 20 hr Experiment Starting with Two Different Initial Concentrations

The different initial concentrations of DBT had an effect on the rate of conversion (or reaction rate) as presented in section 4.2.3. This section will verify this effect before proceeding with major conclusions. The reaction rate was measured for two concentrations: 1 mmole/L and 2.5 mmole/L, for the first 20 hr, during the period of high conversion. The detailed experiment protocol is given in section 3.5.3. The results of absorbance and DBT conversion measurements are presented in Figure 4-25 and Figure 4-26, respectively.

Figure 4-25: Formation of metabolites for 20 hr, based on absorbance at 475 nm. The DBT concentrations were 1 mmole/L and 2.5 mmole/L. Slopes are in AU/min

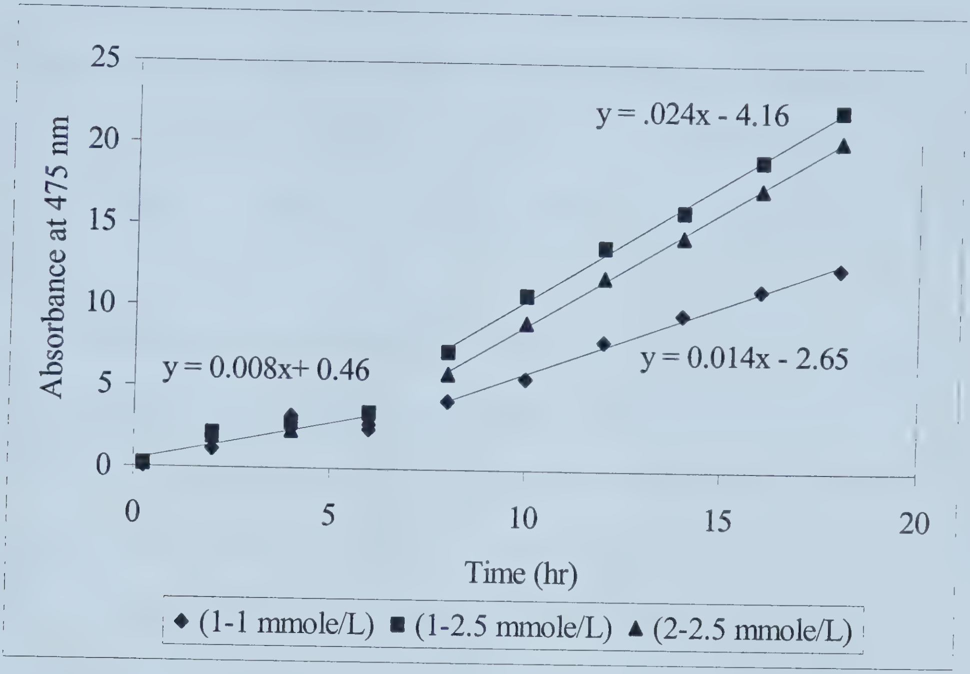
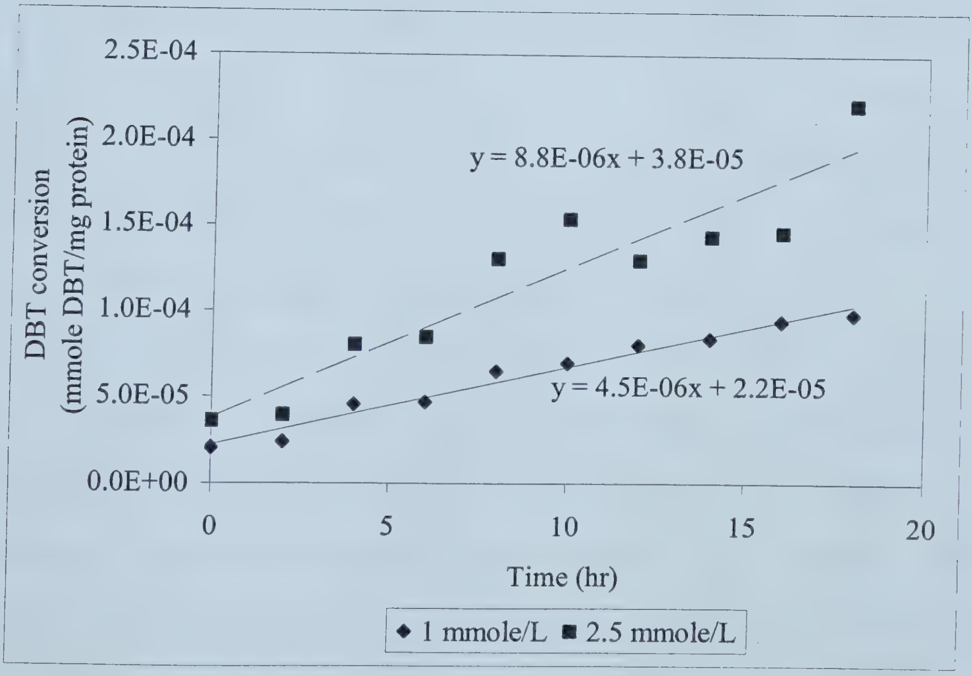


Figure 4-26: DBT conversion with time for initial concentrations of 1 mmole/L and 2.5 mmole/L. The data points are means of replicate flasks



From the data of Figure 4-25, it can be seen that for the initial 8 hours of the experiment, the rate of formation of metabolites the first 3 hr was 0.008 AU/min. For the remaining 10 hr, the rate of formation was 0.014 AU/min for the batch with 1 mmole/L DBT, and for the two batches at 2.5 mmole/L DBT concentration the rates were 0.024 AU/min.

From Figure 4-26, the rates of DBT conversion for the two concentrations were positive and conversion increased linearly with time. The slope of the curve for the 1 mmole/L batch was found to be $4.5 \pm 2.7\text{x}10^{-6}$ µmole/µg protein/hr, and the slope for the 2.5 mmole/L batch was $8.8 \pm 7.9\text{x}10^{-6}$ µmole/µg protein/hr. The two curves were statistically different ($p<0.05$). The difference in slope implies that the conversion rate in the aqueous phase was influenced by concentration of DBT in the hexadecane. The following section will investigate the effect of the organic carrier concentration on the aqueous phase concentration. Meanwhile, the fact that the conversion increased when DBT concentration increased implied that the enzymatic activity was higher than the rate of transfer of DBT into the aqueous phase. To examine if the biocatalyst activity was high and the conversion was limited by mass transfer or partitioning, the biocatalyst was exposed to DBT as a solid suspension and as organic solution.

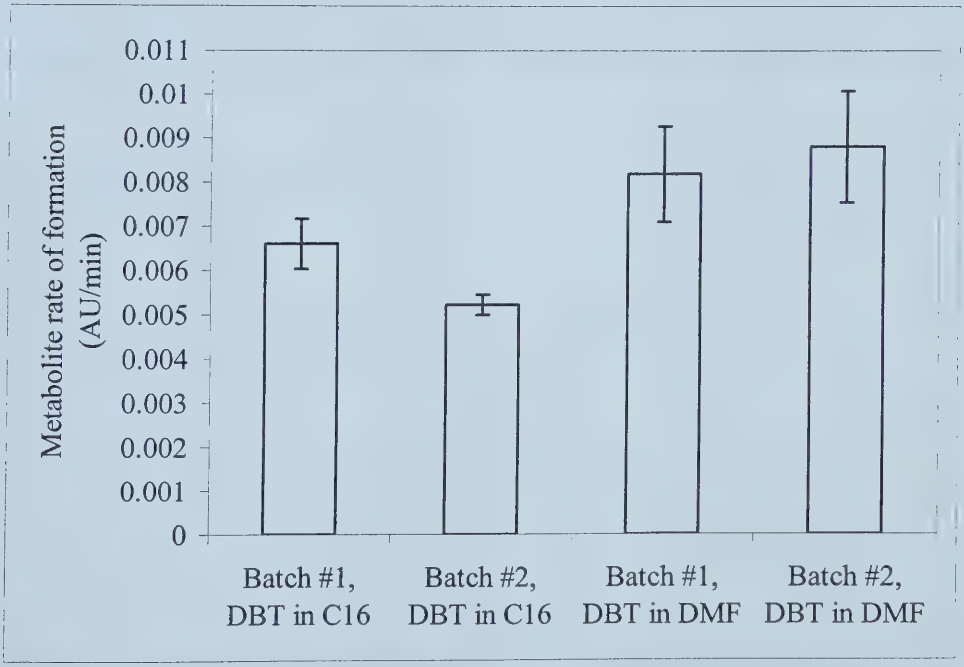
Two 500 mL batches of an induced biocatalyst were prepared, and each was divided into 2x200 mL volume. From each of the two batches, one was exposed to DBT as a suspension of crystal added in DMF, and the other two had DBT dissolved in C_{16}. The two stock solutions had concentration of 0.1 M, where 2 mL was added to 200 mL biocatalyst, making the final concentration 1 mmole/L. The batches had similar amounts

of 2AB. The experiment measured the absorbance at 475 nm for the initial 75 min. The results are shown in Table 4-3, and the rates of formation of the metabolite (AU/min) is presented in Figure 4-27, where the error bar describes the upper and lower 95% confidence intervals. The rates were statistically different between the two treatments, and the DMF treatment showed a higher rate, which implies that the enzymatic system was highly active and that the presence of a non-aqueous liquid phase lowered the biotransformation rate. These results suggest that the system is mass transfer limited, rather than enzymatically limited. The following experiment will illustrate the effect of the organic phase on the dissolution rate of DBT.

Table 4-3: Initial metabolite absorbance at 475nm. The batches were exposed to DBT in a solid suspension form and in an organic solution

	Batch #1		Batch #2	
	$OD_{600} = 3.57$		$OD_{600} = 3.91$	
Time (min)	DBT in C_{16}	DBT in DMF	DBT in C_{16}	DBT in DMF
5	0.084	0.096	----	0.088
10	0.092	0.194	0.076	0.168
20	0.14	0.237	0.139	0.229
27	0.19	0.269	0.171	0.264
32	0.22	0.312	0.201	0.301
40	0.261	0.344	0.234	0.33
47	0.329	0.404	0.274	0.442
55	0.386	0.5	0.314	0.472
65	0.468	0.622	0.36	0.634
75	0.524	0.704	0.428	0.748

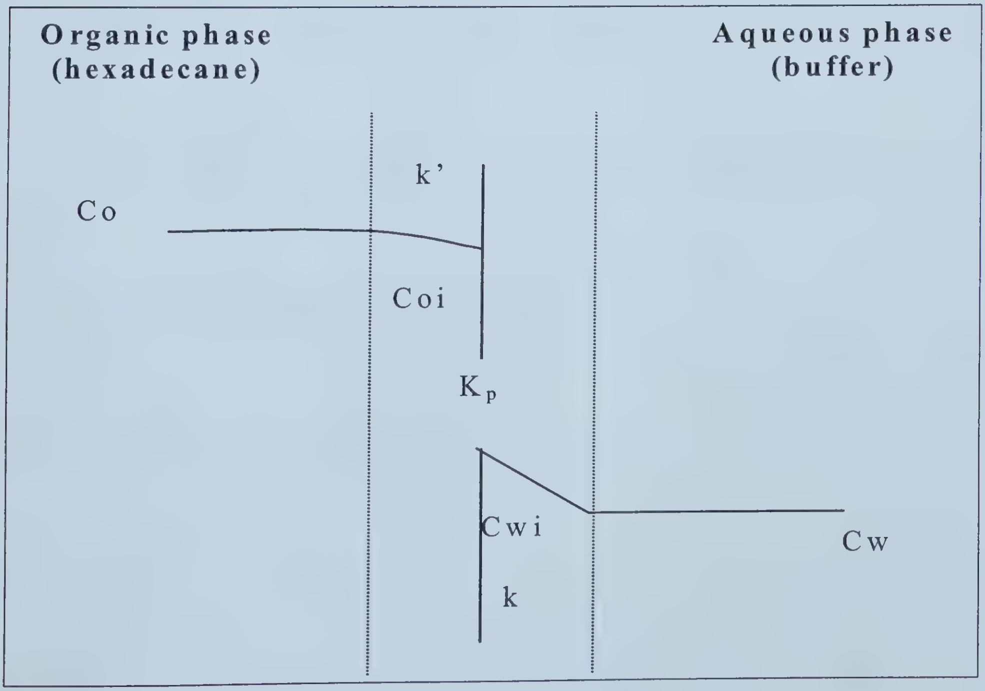
Figure 4-27: Rate of formation of metabolite absorbance at 475 nm, for batches exposed to DBT in a solid suspension form and in organic solution



4.2.4.1 DBT Dissolution from the Organic Carrier Phase

In aqueous-organic phase biocatalyst systems, the substrate diffuses from the organic phase to the aqueous phase, and the microorganisms transform the substrate at the interface and / or in the aqueous phase. Based on the results of section 4.1.1.2 the mutant 21-41 utilized the PAC once it had dissolved in the aqueous phase because the cells did not adhere to the organic layer. Thus, the dissolution rate of DBT from hexadecane into the aqueous phase is described as the mass or moles of DBT transferred into the water per unit of time per unit surface area of the organic phase in contact with the aqueous phase. The diagram in Figure 4-28 presents the flux of DBT from the bulk of the organic phase to the aqueous phase.

Figure 4-28: Concentrations of DBT across the aqueous/organic interface



This flux can be expressed in terms of an overall mass transfer coefficient and a driving force, as shown in Equation (3-4). The units of the variables and the parameters are listed as calculated in this work, and could be transferred to SI units utilizing the appropriate conversion factors.

$$Flux = k'\,(Co - Coi) = k(Cwi - Cw) \qquad \textbf{(3-4)}$$

Where,

Flux = the release of DBT, μmole.m^{-2}.hr^{-1}

$k^{/}$ = overall mass transfer coefficient in the hexadecane phase, m.hr^{-1}

k = overall mass transfer coefficient in the aqueous phase, m.hr^{-1}

Co = organic phase concentration of DBT in the bulk of the hexadecane phase, μmole.mL^{-1}

Coi = organic phase concentration of DBT in equilibrium with the aqueous phase, μmole.mL^{-1}

Cwi = aqueous concentration of DBT at equilibrium with the hexadecane phase, μmole.mL^{-1}

Cw = the aqueous concentration of DBT, μmole.mL^{-1}

The term (Cwi - Cw) is the driving force for DBT to enter the aqueous phase. There is a dependency relationship between the substrate concentration in the aqueous phase and the substrate concentration in the organic phase. This relationship was investigated by measuring the UV absorbance of DBT in the aqueous phase with different initial concentrations in hexadecane. The two-phase system was prepared to reach equilibrium.

Four DBT concentrations in hexadecane were tested; 0.1 M, 0.15 M, 0.2 M and 0.25 M. A total of 12x500 mL Erlenmeyer flasks were prepared, each with 200 mL buffer and 3 flasks prepared at each concentration. Each flask had a 2 mL volume of DBT solution in hexadecane, each from independently prepared concentrated stock solutions. The batches were shaken for 18 hr at 28°C and 200 rpm. The contents of each flask were poured into independent 500 mL separatory funnels, where they were left for 1 hr to separate into two clear distinguishable layers. The UV-spectrophotometer was set to scan wavelengths from 200-400 nm. Samples were withdrawn from the separatory funnels, discarding the first 2 mL. Samples were taken in triplicates. The samples were read at two wavelengths: 232 nm and 284 nm. The former is the absorbance maximum for DBT, from the PAC spectrum. The partitioning of hexadecane to the aqueous phase could not be detected, because it does not absorb UV. The results are presented in Figure 4-29, showing the changes in absorbance of DBT in the aqueous phase at equilibrium as a result of varying DBT concentration in the organic phase.

According to the Beer-Lambert Law in Equation 2, the changes in the solute (DBT) absorbance indicate changes in the solute concentration. Therefore, the absorbance changes shown in Figure 4-29 corresponded to different aqueous concentrations. To identify these concentrations, a stock of concentrated solution of DBT was prepared in 95% ethanol solution, and diluted in the phosphate buffer. The standard curve in Figure 4-30 presents a range of DBT concentration versus absorbance at 232 nm. The corresponding concentrations for the different absorbance shown in Figure 4-29 are presented in Table 4-4 and Figure 4-31. All aqueous concentrations were below the maximum solubility of DBT, which is 1.47 mg/L at 24 °C (Pearlman et al., 1984).

Figure 4-29: Aqueous phase absorbance at 232 nm with varying DBT concentration in hexadecane

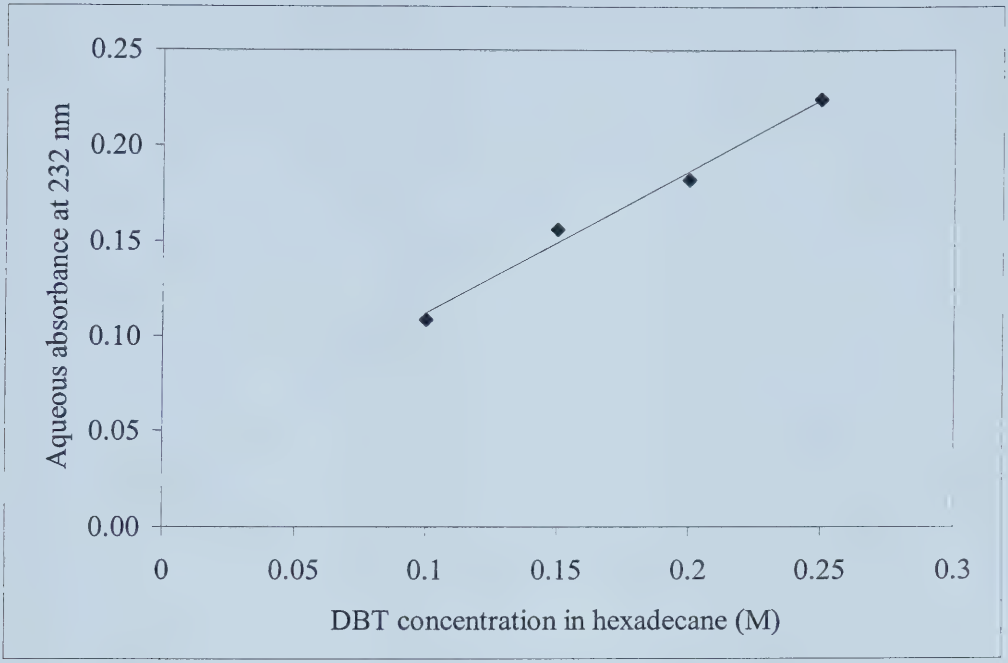


Figure 4-30: Standard curve for DBT solubility in buffer

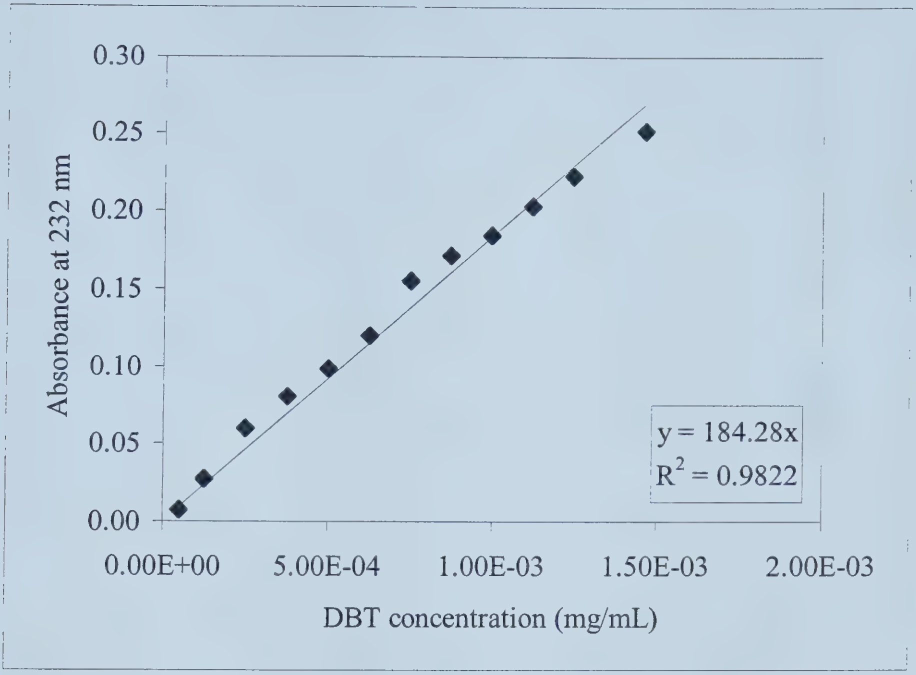
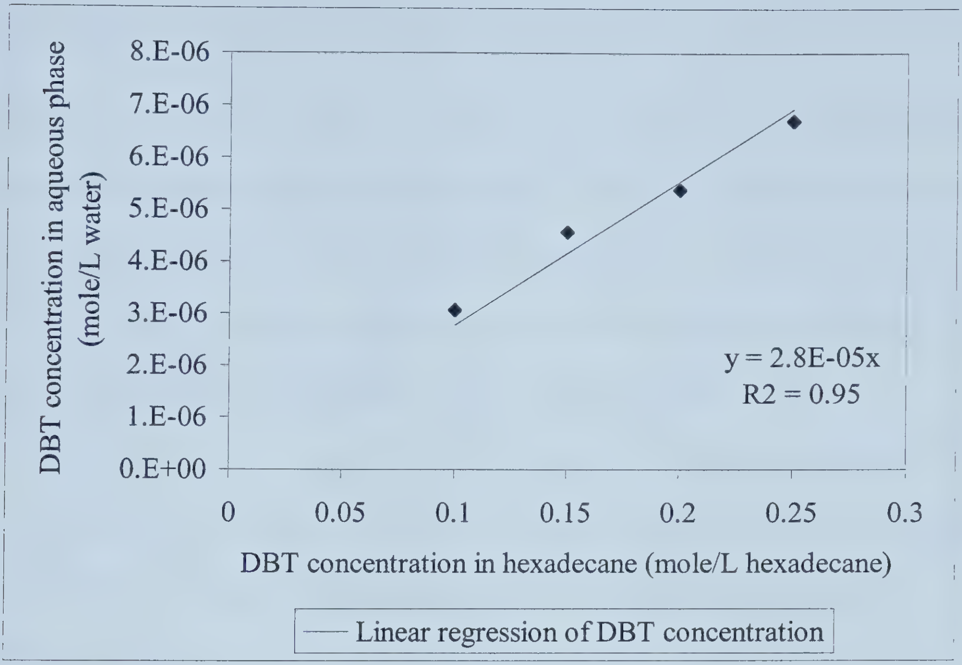


Table 4-4: DBT concentration in aqueous phase with respect to varying DBT concentration in hexadecane

DBT concentration in hexadecane (M)	DBT concentration in hexadecane (mg/L)	DBT concentration in aqueous phase (mg/L)
0.1	18420	0.59
0.15	27640	0.85
0.2	36860	0.99
0.25	46060	1.20

Figure 4-31: DBT concentration in aqueous phase with respect to varying DBT concentration in hexadecane



As shown in Figure 4-31 the DBT equilibrium concentration in water had a linear relation with DBT concentration in hexadecane. Thus, $C^* = \frac{C_o}{K_P}$ where C_o is the bulk concentration of DBT in hexadecane (mole.L^{-1}), C^* is the aqueous concentration of DBT at equilibrium with the hexadecane phase (mole.L^{-1}), and K_P is a dimensionless partition coefficient expressed on a concentration basis, (mole DBT.L^{-1} of hexadecane) / (mole DBT.L^{-1} of water). The experimental value of the DBT partition coefficient was $K_P = 2.8 \times 10^{-5}$ (mole DBT.L^{-1} of hexadecane) / (mole DBT.L^{-1} of water).

The next step was to study the reaction kinetics of the biocatalyst. The method used to determine the reaction rate was the initial rate method. This method examines the initial reaction rate under varying concentration. The next experiment will study 4 different concentrations.

4.2.5 Initial Rate of DBT Conversion as a Function of DBT Concentration

The study of reaction kinetics gives a reaction rate equation, describing the dependency of reaction rate on initial substrate concentration. Kinetic analysis may utilize the initial rate values (Fogler, 1992), therefore, conversion of DBT was measured over the first 4 hr at concentrations of 1 mmole/L, 1.5 mmole/L, 2.0 mmole/L and 2.5 mmole/L. The induced biocatalyst was prepared in 4x650 mL flasks. The pellets were suspended in buffer to an $OD_{600} = 4.7$. The biocatalyst was divided into 30 mL batches representing each time point. There were triplicate or duplicate batches for single time points. The batches had 0.3 mL of hexadecane with DBT at concentrations of 0.1 M, 0.15

M, 0.2 M and 0.25 M, which made the concentration of the total volume being 1 mmole/L, 1.5 mmole/L,2 mmole/L and 2.5 mmole/L. The GC was used to measure the DBT remaining at each time interval, and thus calculate the amount converted within the first four hours.

The data shown in Figure 4-32 present the initial conversion of DBT for the first 4 hr. The initial conversion at time zero showed a value other than zero, which was due to errors in extraction and analysis. The initial rates of conversion shown in Figure 4-33, were calculated by fitting all the experimental conversion data in Figure 4-32 by linear regression for each concentration. The slope of the line fitting the four different rates, as illustrated in Figure 4-33, had a statistically significant positive slope, which indicated a dependency of DBT conversion on the substrate concentration in hexadecane. The biocatalyst enzymatic system has not become saturated, even at the highest concentration used.

Figure 4-32: Initial conversion of DBT during a 4 hr experiment at different concentrations. Initial concentrations ranged from 1 mmole/L to 2.5 mmole/L in biocatalytic system. Data points are means of duplicate or triplicate samples

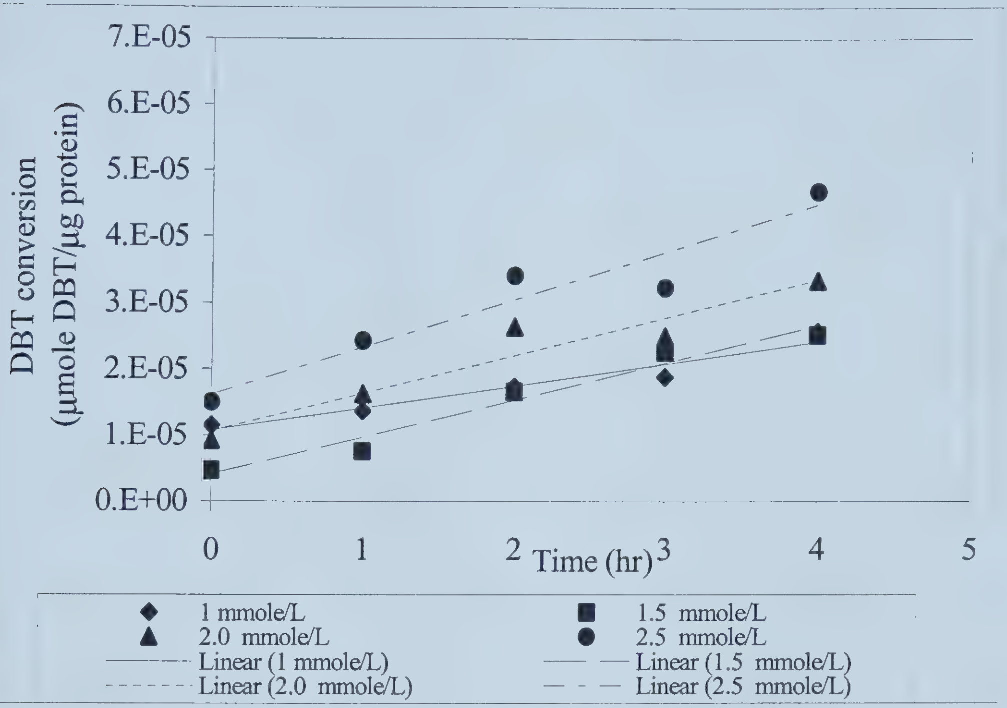
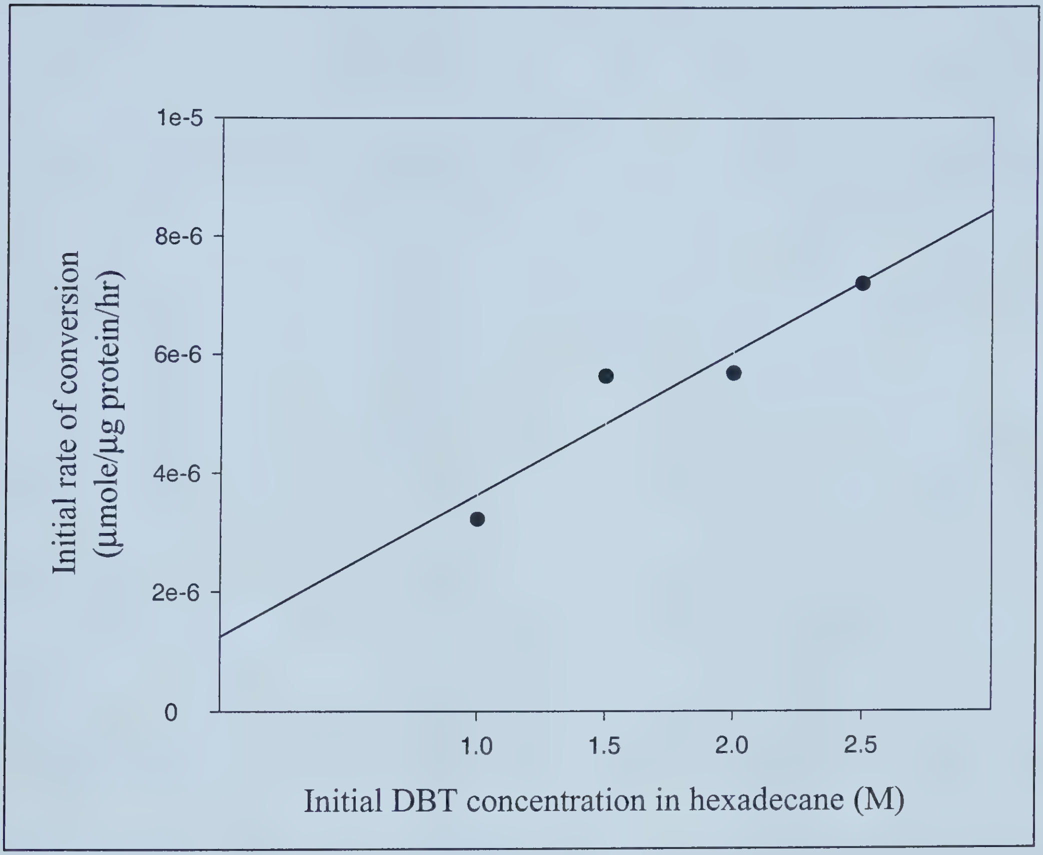


Figure 4-33: Initial rate of conversion with different initial concentrations of DBT during a 4 hr experiment



4.3 Inhibition Due to Metabolites from DBT

According to Monticello et al. (1985) three isolates of *Pseudomonas* species, which oxidized DBT, showed inhibition in the presence of DBT degradation products. The inhibition affected the isolate growth and the further oxidation of DBT to water-soluble products. Therefore, one of the objectives of this work was to study the effect of DBT metabolites formed by mutant 21-41 on the further oxidation of DBT.

The following experiments were designed to identify if the DBT metabolites were inhibiting the biocatalyst activity, and thus reducing the reaction rate. The effect of the metabolites on the biocatalyst activity was determined by adding different volumes of metabolite solution to a fresh (unused) biocatalyst, then measuring the conversion of DBT. The first experiment varied the volume of the induced biocatalyst, the metabolite aqueous solution and the substrate volume. In subsequent experiments, several ratios were maintained constant: (1) $\frac{Biocatalyst\ \ cells}{aqueous\ volume}$, (2) $\frac{Biocatalyst\ \ cells}{Substrate\ organic\ volume}$, and (3) $\frac{Substrate\ organic\ volume}{aqueous\ volume}$. The main variable was the volume of the metabolite solution at the initial time. The metabolites were diluted with phosphate buffer to keep the biocatalyst volume constant.

4.3.1 Preparing Stock Solution of Metabolites

The stock solution of metabolites was prepared by using a sufficient volume of induced biocatalyst. The appropriate amount of (0.1 M) substrate and 2AB were added to the batch, and then it was shaken for 24 hr at 28 °C and 200 rpm. The biocatalyst cell

pellets and the excess substrate were removed by centrifugation at 16,300xg and 4°C. The aqueous supernatant containing the metabolites was collected and extracted twice with 50 mL pentane to remove residual hexadecane and DBT. The aqueous metabolite solution was drained, since it formed the extraction bottom layer. A syringe permitted the flow of nitrogen gas for 10 min, allowing the removal of excess pentane. The stock metabolite solution was wrapped with aluminum foil and stored at 4 °C. Before using the stock solution, 1 mL samples were taken to measure an equivalent absorbance at 475 nm of 12. This absorbance corresponded approximately to a 55% DBT conversion, based on data in section 4.2.1 and 4.2.3.

4.3.2 Effect of DBT Metabolites on the Conversion of DBT

A 200 mL batch of induced biocatalyst was concentrated in 140 mL of buffer. A 20 mL volume of the concentrated solution was transferred to 7 independent batches. Volumes from the stock metabolites and buffer were added to bring the total biocatalyst volume to 200 mL. The second inducer 2AB and substrate volumes were 0.2 mL, and 2 mL, respectively. The different volumes of metabolites and buffer are shown in Table 4-5.

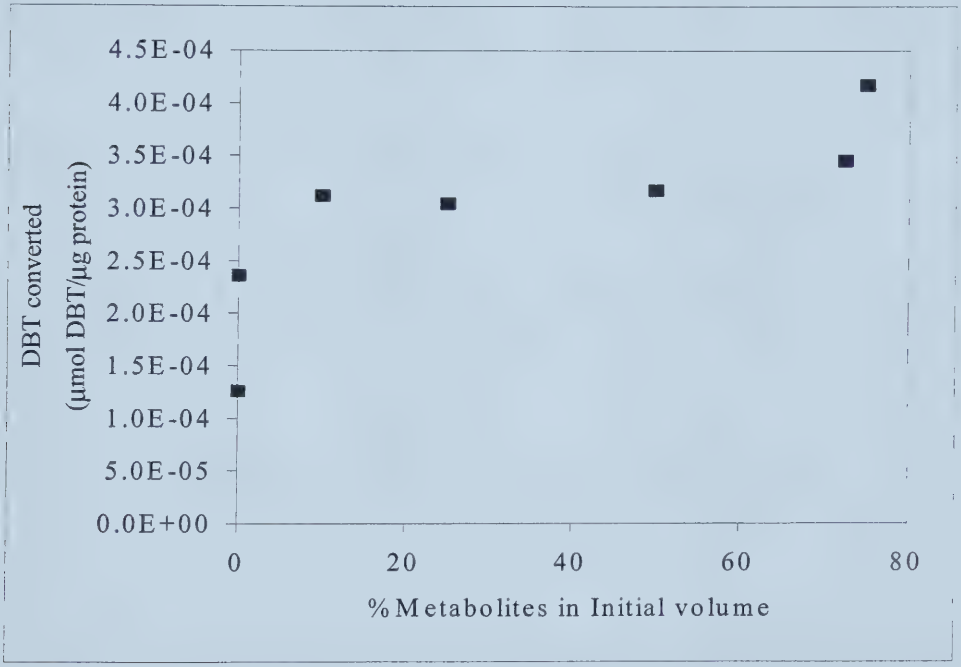
The batches were shaken at 200 rpm, at 28 °C for 18 hr. The choice of 18 hr was to avoid the high conversion of DBT after 24 hr, and therefore allow detection of variations in conversion. Figure 4-34 presents the results of the DBT converted as initial concentration of the metabolites increased. The DBT conversion was very similar between the different batches. The major difference was between the two control batches (i.e. the 0% initial metabolites). These data suggested that DBT metabolites were not inhibiting mutant 21-41 from further conversion of DBT, within the 18 hr experimental

period. The results were not expected, therefore another run had to be conducted. Other reasons for repetition were that the biomass was diluted more than the regular 200 mL biocatalyst by 10-fold, and the initial metabolite concentration was below 90%. Therefore in the following experiment the stock biocatalyst was concentrated to an initial OD_{600} of 3, and a larger volume of metabolite stock solution was prepared.

Table 4-5: Experimental design for testing the effect of varying initial metabolite volume

Initial metabolite concentration, %	0	10	25	50	72.5	75
Fresh cell volume (mL)	20	20	20	20	20	20
Metabolite volume (mL)	0	20	50	100	145	150
Buffer volume (mL)	180	160	130	80	35	30
Biocatalyst total volume (mL)	200	200	200	200	200	200
Substrate volume (mL) (0.1 M) DBT in hexadecane	2	2	2	2	2	2

Figure 4-34: DBT conversion over 18 hr as a function of initial metabolite volume



4.3.3 Effect of DBT Metabolites on the Conversion of DBT at Higher Biomass Concentration

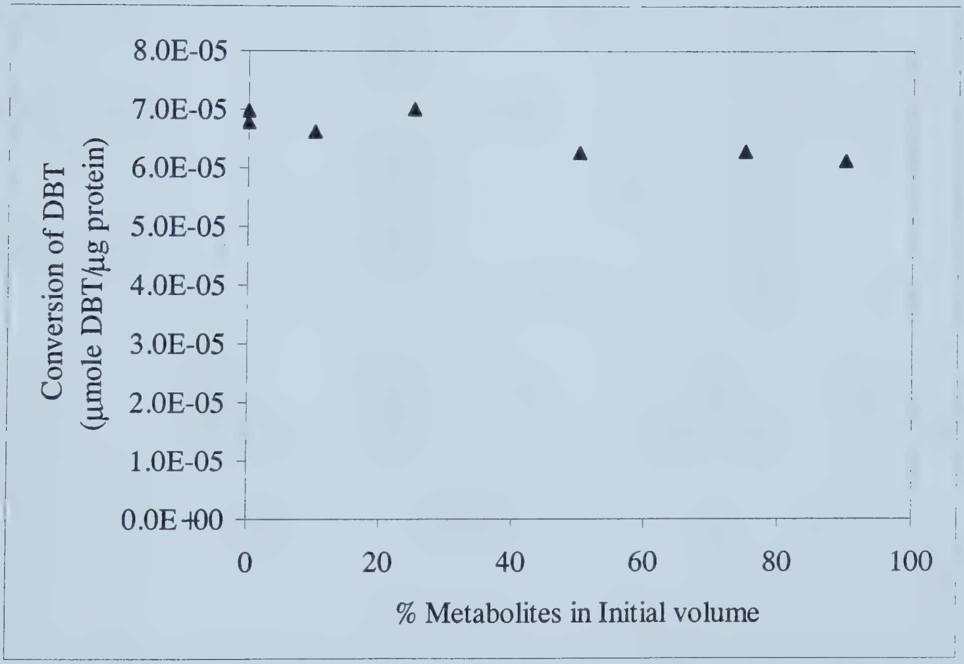
The biocatalyst batches in this experiment had a higher biomass concentration than the previous experiment, and the percent of metabolite solution increased to 90% rather than 75%. Three batches were prepared each with 700 mL of induced biocatalyst, which was centrifuged to recover the cells. The biocatalyst pellets were resuspended into a 200 mL volume of buffer. Thus the main batch of induced biocatalyst was prepared as a concentrated suspension, from which 20 mL was transferred to seven independent batches, each of which had an initial $OD_{600} = 3$. The batch volume was completed to 200 mL, as shown in Table 4-6. Two-tenths milliliter of 2AB acid was added to each batch, and then the batches were shaken at 200 rpm, 28 °C for 18 hr.

The data in Figure 4-35 did not show a significant difference in DBT conversion as the initial metabolite concentration increased. This result confirms that the metabolites of DBT were not inhibitory under the conditions used. The 10-fold variation in the conversion rate between the first and second trial was due to difference in biomass. However the conclusion from the two experiments is valid and comparable.

Table 4-6: Experimental design for testing the effect of varying initial metabolites volume

Initial metabolite concentration, %	0	0	10	25	50	75	90
Fresh cells volume (mL)	20	20	20	20	20	20	20
Metabolites volume (mL)	0	0	20	50	100	150	180
Buffer volume (mL)	180	180	160	130	80	30	0
Biocatalyst total volume (mL)	200	200	200	200	200	200	200
Substrate volume (mL) (0.1 M) DBT in hexadecane	2	2	2	2	2	2	

Figure 4-35: DBT conversion over 18 hr as a function of initial metabolite volume



4.4 Rejuvenating the Biocatalyst

The addition of energy sources to sustain the energy pool in the resting cells is a standard procedure in working with whole cells as biocatalyst. For example, a *Pseudomonas saccharophila* P15 strain grows on phenanthrene had succinate added as a primary energy source to all experimental samples and controls, to exclude the possibility that low activity might be caused by endogenous energy losses over the incubation period (Chen & Aitken, 1999). Similarly, the addition of energy sources can sustain the cofactors for enzymes. For example, Bianchi et al. (1997) was investigating the oxidation of a series of three ring heterocyclic compounds by the naphthalene dioxygenase of *Pseudomonas fluorescens* TTC1 (NCIMB 40605). All the oxidation reactions were carried out using *P. fluorescens* TTC1 resting cells. The biotransformations were performed by resuspending the cells in a minimal medium with glucose added as a cosubstrate to regenerate the cofactors.

Since the biocatalyst in this study used cells in the resting state, rejuvenation experiments became one of the research objectives. Before proceeding with rejuvenation, it was necessary to find the appropriate sequence for adding the energy sources. The first attempt was to add the energy source at the initial time.

4.4.1 Simultaneous Supply of Glucose and DBT

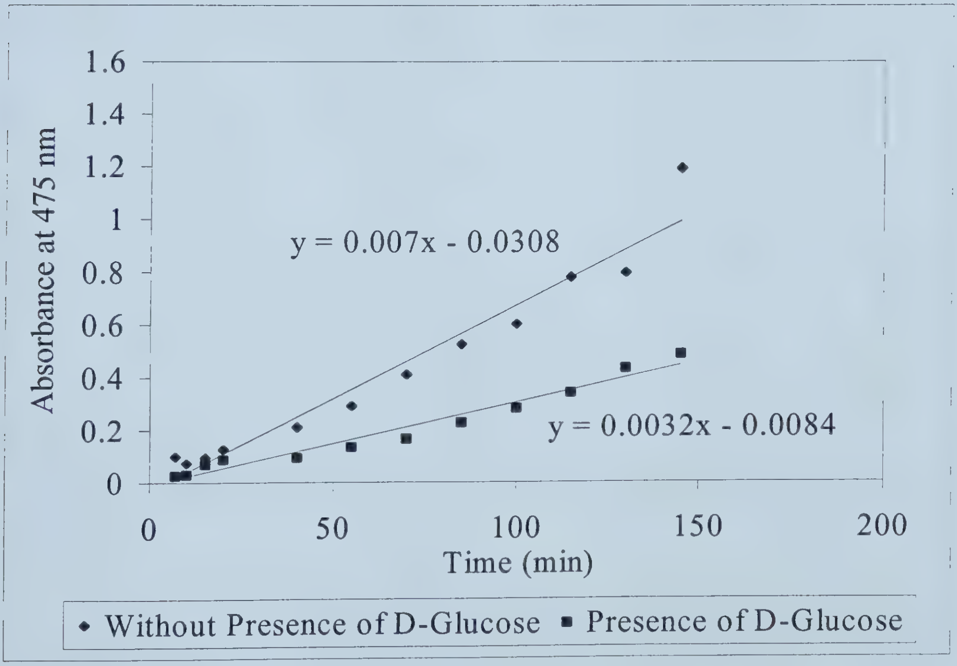
The first approach to keep the biocatalyst activity high was the addition of energy sources at the start of the reaction time. By providing an energy source without supplying the entire range of nutrients needed for growth, there would be constant flow of energy but growth of the cells would be suppressed.

The results for DBT conversion in the presence of glucose are shown in Table 4-7. Detailed methods are given section 3.6.1. The colored metabolites showed a lower rate of formation in the presence of glucose than in the control batch without glucose. By plotting the data, as in Figure 4-36, the rate of DBT conversion could be calculated as absorbance unit per min (AU/min). This conversion gives a direct indication of enzyme activity over the total time of 145 min. The enzyme activity for the batches with and without glucose were 0.003 AU/min and 0.007 AU/min, respectively. These results implied a catabolic repression effect by adding glucose in the presence of the substrate. Therefore, the biocatalyst rejuvenation should be done as a separate step or a separate unit for large-scale production.

Table 4-7: Effect of glucose addition on the metabolite formation

Time (min)	Without presence of glucose (Absorbance at 475 nm)	With presence of glucose (Absorbance at 475 nm)
7	0.10	0.03
10	0.07	0.03
15	0.10	0.07
20	0.13	0.09
40	0.21	0.10
55	0.29	0.14
70	0.41	0.17
85	0.53	0.23
100	0.60	0.28
115	0.78	0.34
130	0.80	0.44
145	1.19	0.49

Figure 4-36: Effect of glucose addition on the metabolite formation



4.4.2 Rejuvenation of Exhausted Biocatalyst

This experiment was designed to study the rejuvenation of the exhausted biocatalyst, by adding TSB media and glucose as energy sources or dithiothreitol and ascorbic acid as reducing agents. The additions of energy sources were not intended to permit formation of new bacteria; thus the exposure time was less than the doubling time or generation time.

4.4.3 Rejuvenation Experiment with a 48 hr Exhausted Biocatalyst

This rejuvenation experiment was done with a biocatalyst used for 24 hr. The energy sources added were TSB media and glucose. The experimental protocol is illustrated in Figure 4-37 where each run was for 24 hr. Details of the method are given in section 3.6.2. The DBT conversion results, as shown in Table 4-8, showed that rejuvenation was possible by adding energy sources to an exhausted biocatalyst.

Figure 4-37: Rejuvenation experimental procedure

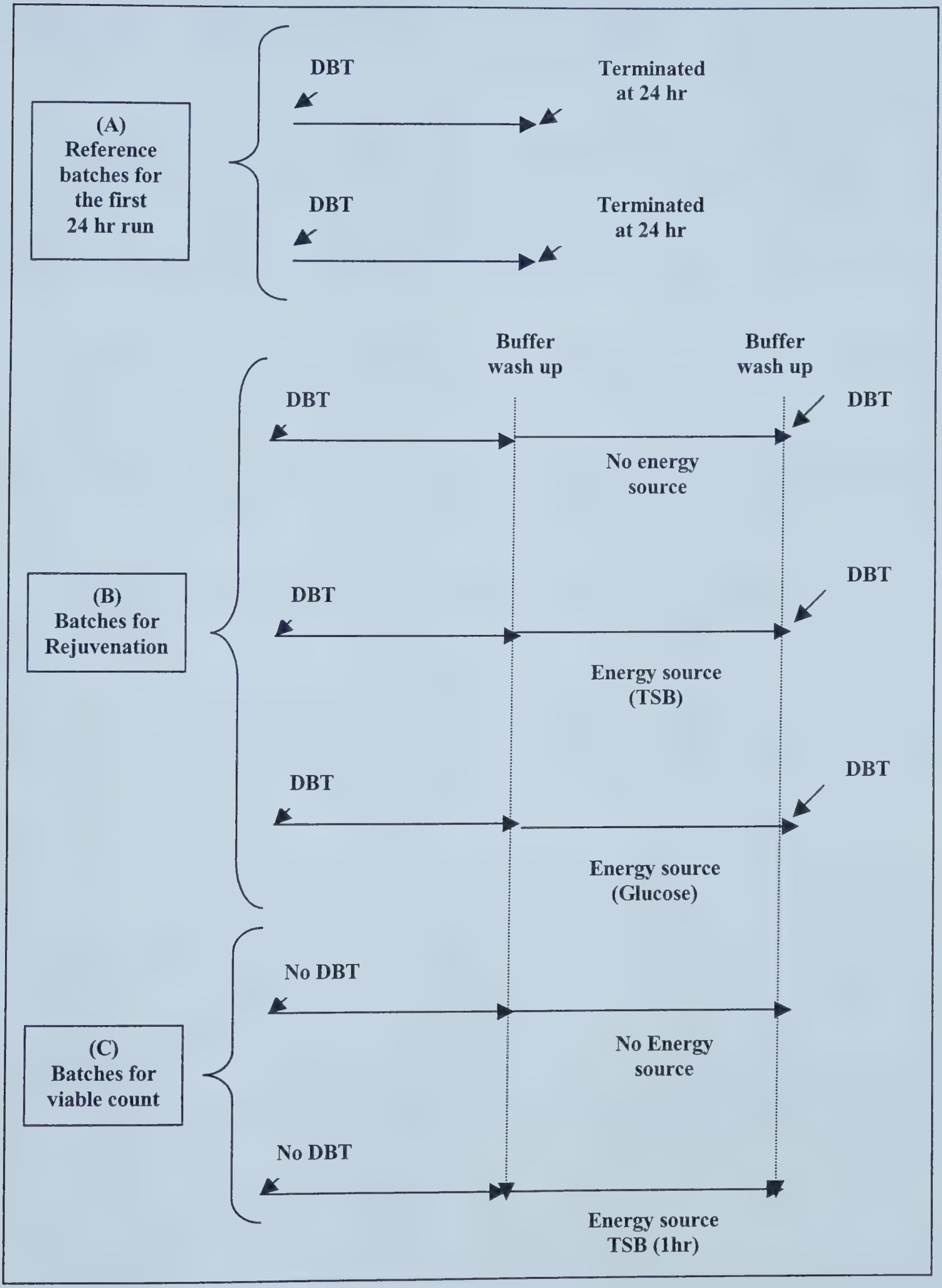

(A) Reference batches for the first 24 hr run
DBT
Terminated at 24 hr
DBT
Terminated at 24 hr
Buffer wash up
Buffer wash up
(B) Batches for Rejuvenation
DBT
DBT
No energy source
DBT
DBT
Energy source (TSB)
DBT
DBT
Energy source (Glucose)
(C) Batches for viable count
No DBT
No Energy source
No DBT
Energy source TSB (1hr)

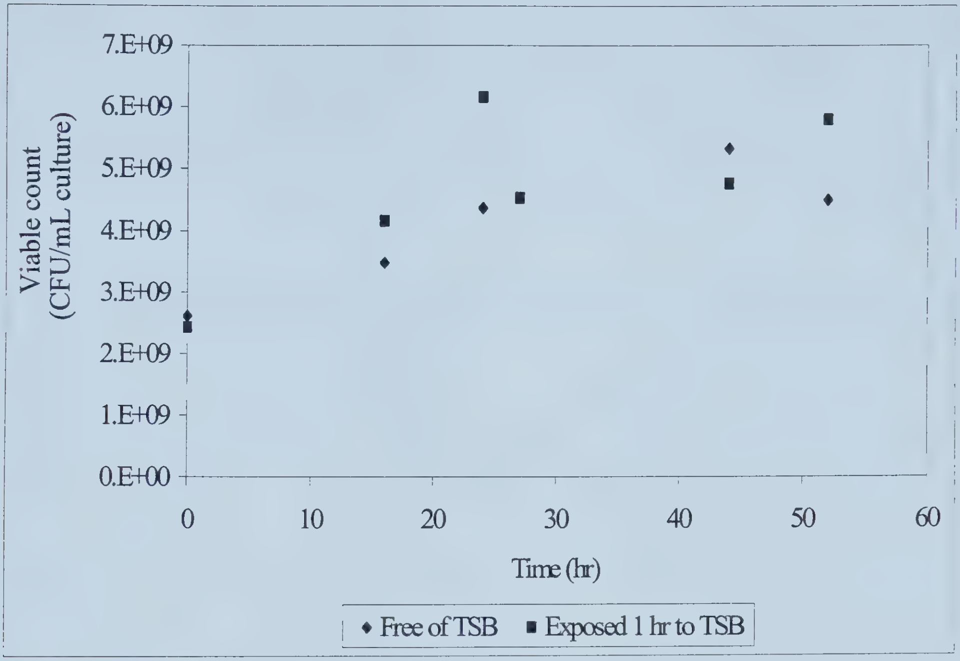
Table 4-8: DBT conversion after rejuvenation of the biocatalyst

Reaction Time (hr)	Energy Source	%Conversion	DBT Conversion (μmole DBT/μg protein)	Rate of DBT conversion (μmole DBT/μg protein/hr)
First-24 hr	---	72	8.75 E-5	3.65 E-6
First-24 hr	---	79	8.05 E-5	3.35 E-6
Second-24 hr	Buffer	7	3.10 E-5	1.29 E-6
Second-24 hr	TSB	17	3.36 E-5	1.40 E-6
Second-24 hr	Glucose	26	5.26 E-5	2.19 E-6

The batches in group C were used to analyze the viable counts. The two batches were centrifuged, washed and shaken as the other batches. After 24 hr one of the batches was exposed to 30 mg/mL TSB media for 1 hr, after which it was centrifuged and washed. The two batches were independently suspended in 200 mL buffer, and returned for incubation at 28 °C for 24 hr and 200 rpm. At each step a 1 mL sample was taken from each batch for viable count measurement. The results for this group are shown in Figure 4-38. The viability did not show significant changes with time, thus the reduction in activity was not due to reduction in viability. This conclusion is consistent with the results in section 4.1.1.3.

The addition of energy source to spent biocatalyst for a brief period showed promising results, therefore a series of experiments was conducted to understand and optimize the rejuvenation conditions. Firstly, it was necessary to monitor the consumption rate of the energy source, and compare its value for an exhausted biocatalyst with a non-exhausted one. Glucose was chosen as the first energy source to study.

Figure 4-38: Viable count measurement for the rejuvenation experiment



4.4.3.1 Glucose Consumption Rate for Exhausted Biocatalyst

To monitor the rate of consumption of the energy source, the biocatalyst was used for 24 hr, then different glucose concentrations were added (0.1 mg/mL and 1 mg/mL). A control set of cells was used to compare its rate of consumption with the exhausted biocatalyst.

The experimental plan is illustrated in Figure 4-39, and detailed method is given in section 3.6.3. The results obtained for the two concentrations of 0.1 mg/mL and 1 mg/mL are presented in Figure 4-40 and Figure 4-41, respectively. The glucose consumption rate was similar for batches with and without pre-exposure to DBT, and so was the extent of consumption, which demonstrated the ability of the biocatalyst to rapidly take up glucose. The lower concentration was consumed after only 2 hrs. Therefore for rejuvenation of the biocatalyst, it is recommended to add glucose concentrations above 0.1 mg/mL, such that it is not a limiting factor. These results showed that glucose or other energy sources would be consumed in a period shorter than the mutant generation time depending on the concentrations selected.

Figure 4-39: Glucose consumption experimental design

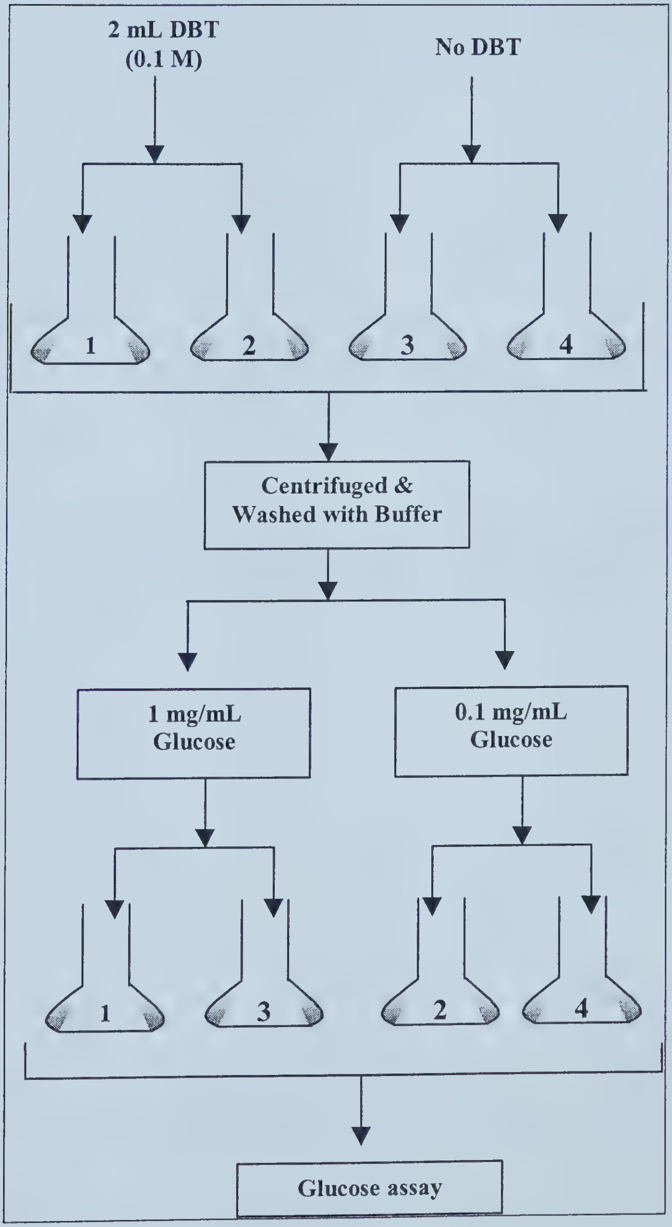

2 mL DBT
(0.1 M)
No DBT
1
2
3
4
Centrifuged &
Washed with Buffer
1 mg/mL
Glucose
0.1 mg/mL
Glucose
1
3
2
4
Glucose assay

Figure 4-40: Glucose consumption by biocatalyst with initial glucose concentration of 0.1 mg/mL. One batch was exposed to DBT

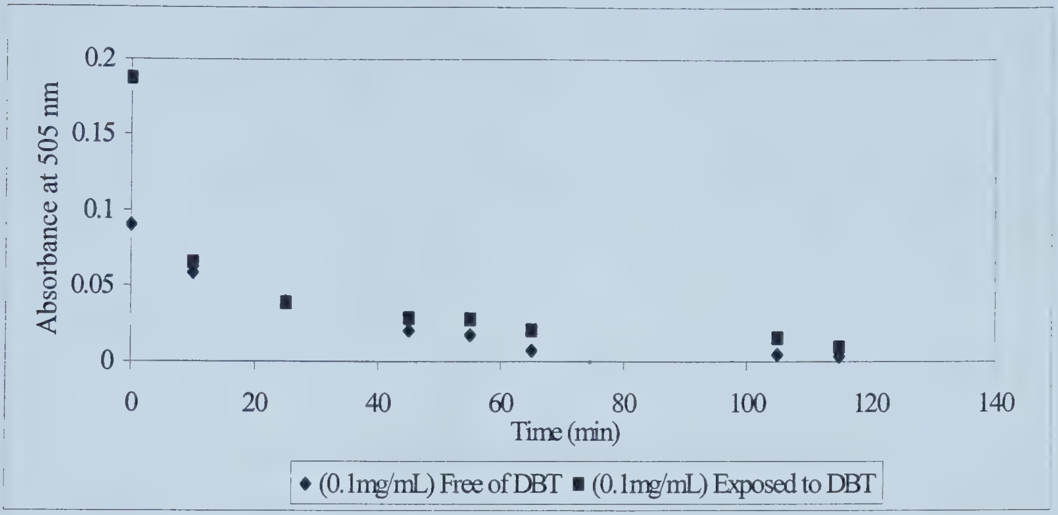
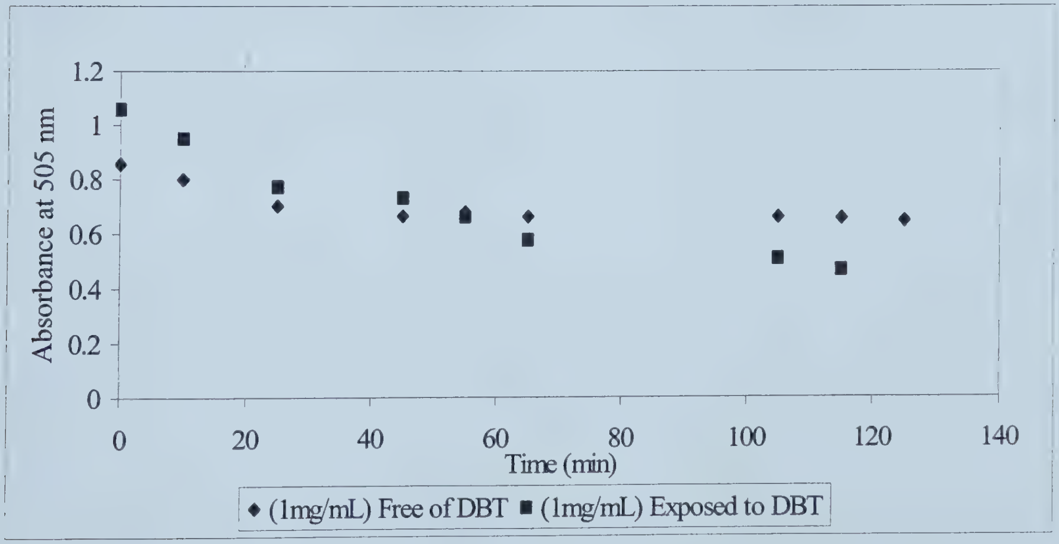


Figure 4-41: Glucose consumption by biocatalyst with initial glucose concentration of 1 mg/mL. One batch was exposed to DBT



4.4.4 Rejuvenation Experiment with a 72 hr Exhausted Biocatalyst

The current experiment investigated rejuvenation of the biocatalyst after three experimental cycles. Each cycle was for 24 hr, after which the biocatalyst was washed, resuspended in buffer and then the substrate was added. Thus, the duration of the cycles was 48, 72 and 96 hr. The experimental procedure is presented in Figure 4-42. Details of the method are given in section 3.6.4.

The results for changes in biomass concentration are presented in Table 4-9 to Table 4-12. These results showed a reduction in total protein content, which was used in calculating specific DBT conversion. However, the results for the biocatalytic conversion did not show significant reduction between the 48, 72 and 96 hr batches, as presented in Table 4-13. This sustained activity was not expected, and thus a repeat experiment was conducted to ensure the validity of the current results before drawing any conclusions.

Figure 4-42: Procedure of the rejuvenation experiment

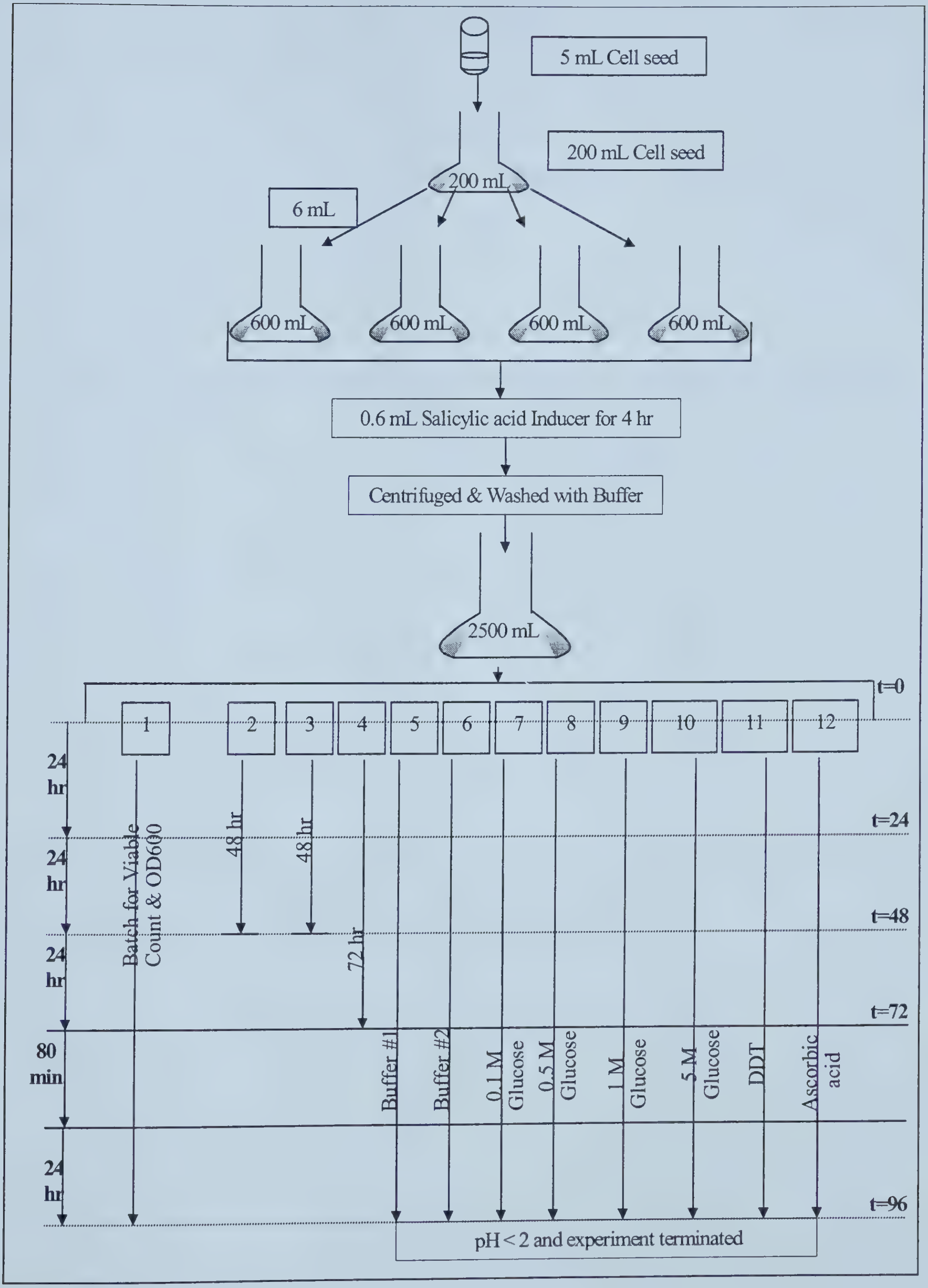

5 mL Cell seed
200 mL Cell seed
200 mL
6 mL
600 mL
600 mL
600 mL
600 mL
0.6 mL Salicylic acid Inducer for 4 hr
Centrifuged & Washed with Buffer
2500 mL
t=0
1
2
3
4
5
6
7
8
9
10
11
12
24 hr
24 hr
24 hr
80 min
24 hr
t=24
t=48
t=72
t=96
Batch for Viable Count & OD600
48 hr
48 hr
72 hr
Buffer #1
Buffer #2
0.1 M Glucose
0.5 M Glucose
1 M Glucose
5 M Glucose
DDT
Ascorbic acid
pH < 2 and experiment terminated

Table 4-9: OD_{600} measurements from flask #1 during the rejuvenation experiment

Time (hr)	OD_{600}
0	3.6
24	2.7
48	2.6
72	2.2
96	2

Table 4-10: Viable count measurements from flask #1 during the rejuvenation experiment

Time (hr)	Total number of colonies (CFU / mL culture)
0	5.4 E9
24	6.8 E9
48	5.4 E9
72	7.1 E9
96	6.7 E9

Table 4-11: Total protein content from flask #1 during the rejuvenation experiment

Time (hr)	Total μg protein
0	2.2 E6
24	2.1 E6
48	1.8 E6
72	1.6 E6
96	1.3 E6

Table 4-12: Equivalent absorbance from diluted samples at the end of each 24 hr period. The data are averaged results from remaining flasks

Time (hr)	Metabolite absorbance at 475 nm
24	14
48	16
72	17

Table 4-13: DBT conversion after 48-72 and 96 hr's. After 72 hr, several batches were exposed to varying energy or reducing sources for 80 min, and one batch was a control. The DBT conversion values were for only a 24 hr period

Parameter – Time (hr)	Average DBT Conversion (μmole DBT/μg protein)
Second-24 hr	6.7 E-5
Third-24 hr	1.0 E-4
Fourth-24 hr-(control) buffer	1.0 E-4
Fourth-24 hr-(0.1 mg/mL) glucose	1.1 E-4
Fourth-24 hr-(0.5 mg/mL) glucose	1.0 E-4
Fourth-24 hr-(1 mg/mL) glucose	1.1 E-4
Fourth-24 hr-(5 mg/mL) glucose	9.6 E-5
Fourth-24 hr-(0.154 mg/mL) dithiothreitol	1.0 E-4
Fourth-24 hr-(0.176 mg/mL) ascorbic acid	8.4 E-5

4.4.5 Reuse of Biocatalyst

The previous experiments showed variable performance when the biocatalyst was reused. Therefore, additional experiments were performed where the biocatalyst was reused repeatedly with removal of the DBT metabolites before each stage. Each stage of the experimental runs was for 24 hr and the number of stages were 2, 3 and 4, i.e. 48, 72 and 96 hr total duration, respectively. This experiment contained two independent batches, with each receiving the same treatment. Details of the method are given in section 3.6.5. The results of OD_{600}, viable counts, total protein content, and absorbance of metabolite at 475 nm are presented in Table 4-14 to Table 4-17.

There were no significant changes in the total viable counts Table 4-15, which emphasizes that variations in the catalyst activity were not due to changes in biocatalyst viability. However, the results for total protein content, shown in Table 4-16, did show a reduction along with the OD_{600} measurement Table 4-14. This result suggests that there was loss in the amount of biomass during the centrifuging and washing technique. Table 4-17 shows the average absorbance at 475 nm at the end of each 24 hr run. These results, which indicate total metabolite concentration, show no reduction in catalytic activity.

Figure 4-43 shows the rate of DBT conversion for each batch versus cumulative moles of DBT converted for a total time of 96 hr. The conversion rate was constant within experimental error. The error considered the standard deviation between the four 200 mL volume batches, which were from the two independent main batches. These results were encouraging as they showed high, sustained productivity. The total amount of DBT converted in the 96 hr experiment averaged $5x10^{-4}$ µmole DBT/µg protein.

Table 4-14: OD_{600} during the experiment with reuse of biocatalyst

Time (hr)	OD_{600} Batch #1	OD_{600} Batch #2
0	3.6	3.7
24	2.6	2.4
48	2.0	2.3
72	2.1	2.0

Table 4-15: Viable count during the experiment with reuse of biocatalyst

Time (hr)	Total number of colonies (CFU / mL culture) Batch #1	Total number of colonies (CFU / mL culture) Batch #2
0	Too numerous to count	2.8 E9
24	3.3 E9	4.0 E9
48	2.9 E9	4.8 E9
72	3.3 E9	3.8 E9

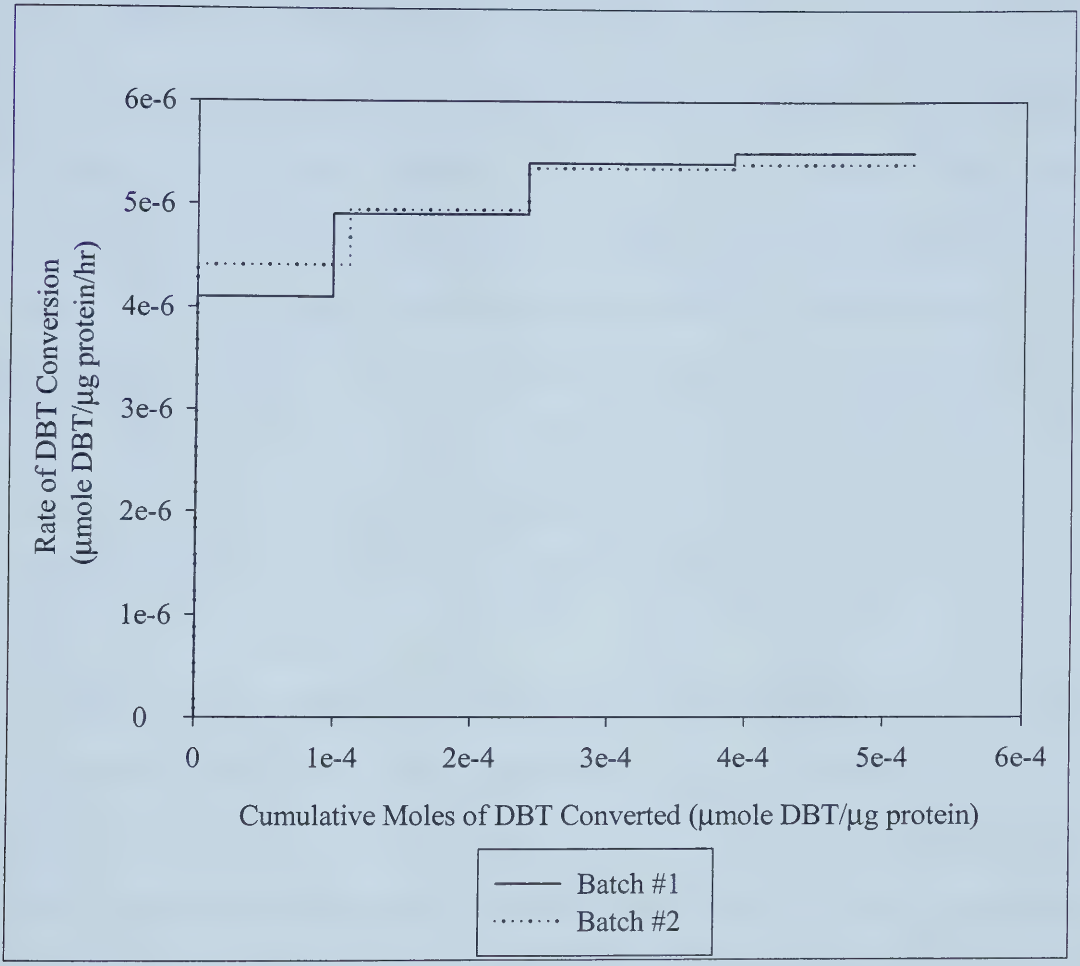
Table 4-16: Total protein content during the experiment with reuse of biocatalyst

Time (hr)	Total μg protein Batch #1	Total μg protein Batch #2
0	1.66 E6	1.56 E6
24	1.36 E6	1.36 E6
48	1.22 E6	1.23 E6
72	1.18 E6	1.16 E6

Table 4-17: Equivalent absorbance of metabolite at the end of each 24 hr cycle

Time (hr)	Metabolite absorbance at 475 nm
24	14
48	16
72	17

Figure 4-43: Rate of DBT conversion versus cumulative conversion, the data represent two independent batches



5.0 DISCUSSION

5.1 Kinetics of Metabolite Formation

The data in Figure 4-14, Figure 4-23, and Figure 4-25 presented changes in the rate of formation of the colored metabolite for the 28 hr, 51 hr, and 18 hr experiment, respectively. The rates were calculated from the slopes as change in absorbance unit per min (AU/min). The changes in slope for the measurements at the same wavelength imply involvement of structures with different extinction coefficients. The expected metabolite had an open ring structure, which later forms some unknown side products. The metabolite as shown in Figure 4-20 had slightly varying chemical structure. The ratios of these structures were varying with time, which explains the change in slope at absorbance 475 nm.

The changes in slope hindered attempts to calculate the molar extinction coefficients. As the molar extinction coefficients of the DBT metabolites were not known, the DBT rate of conversion was expressed as moles of DBT converted per protein content per time rather than as moles products formed per protein content per time (Foght & Westlake, 1990). The moles of DBT converted were monitored by GC-FID and showed consistent repeatability.

From the GC-MS analysis it was confirmed that *Pseudomonas fluorescens* LP6a mutant 21-41 biotransformed DBT via the pathway reported by Kodama et al. (1973). In support of this observation, Foght and Westlake (1996) showed that strain LP6a has a 63 kb plasmid (pLP6a) carrying genes encoding enzymes necessary for PAC-degradation, which hybridizes to the classical naphthalene degradative plasmids NAH7 in the

degradative and non-degradative regions. The NAH7 codes for enzymes that degrade the PAC via the cleavage of a side ring. Denome et al. (1993) showed that for *Pseudomonas* strain C18 the upper naphthalene catabolic plasmid (*nah-1*) had a genetic sequence identical to the one for DBT oxidation (i.e. the DOX genes) with the exception of one gene. These results indicated that a single genetic pathway controls the metabolism of DBT, naphthalene, and phenanthrene in that organism.

Meanwhile, the Kodama pathway, as shown in Figure 2-11, has HFBT as the subsequent product after the presumed end product of mutant 21-41. This product that has a molecular weight of 181 (Bressler & Fedorak, 2001) was not detected in the GC-MS total ion chromatography data. However, an abundance of higher molecular weight structures were found such as 250, 262, and 264. This observation verifies that mutant 21-41 has a blocked degradation pathway.

5.2 Kinetics of DBT Conversion

The experimental system was designed as two immiscible phases for future studies of the biocatalyst activity in presence of diesel fuel. Thus DBT was dissolved in hexadecane, which was chosen for its low volatility, low toxicity, and also for the inability of the mutant to degrade it. Indeed, the identity of the non-aqueous liquid phase is also important for both biotransformation and partitioning. For example, a test with 10 of 11 *Pseudomonas* sp. showed that the rates of mineralization and partitioning of phenanthrene to water from hexadecane and HMN was 2-10 times higher than the non-alkane non-aqueous phase liquid (NAPL) (Efroymson & Alexander, 1994).

When the biocatalyst was exposed to DBT as a suspension of solids, it showed higher rate of formation of the metabolites, whereas with the same concentration of DBT in C_{16}, the system showed a lower rate, as shown in Figure 4-27. By studying DBT dissolution from the hexadecane, it was found that the concentrations in the aqueous phase fall below the solubility of DBT in water, as shown in section 4.2.4.1. Efroymson and Alexander (1995) showed that a NAPL might sequester a large fraction of a hydrophobic pollutant away from the aqueous phase, causing low aqueous phase concentrations. The concentration of organic solutes partitioning from NAPLs was observed to be low. For example, the organic solute partitioning from NAPLs to groundwater are often found to be 10 times lower than their solubilities in water. Since the biocatalyst showed higher activity in the presence of solid DBT in comparison to DBT in hexadecane, we concluded that the rate of partitioning is lower than the rate of biotransformation. Thus, the system is believed to be mass transfer limited rather than kinetically limited, within the range of substrate concentration used.

Several models have been proposed to study the disappearance of organic compounds due to mineralization by mixed cultures from the environment or by pure cultures. There are several factors that affect the validity of these models, for example, time for induction of the active organisms, the accumulation of toxins produced, depletion of inorganic nutrients or growth factors, the presence of other compounds, and the abundance of the active organisms (Simkins & Alexander, 1984). In this study some of these factors were kept approximately constant and/or eliminated, and the developed model was designed to consider variability of reaction rate as a function of initial substrate concentration.

The present model, which predicts reaction rate of DBT in the presence of organic phase, is based on the following assumptions: (1) The enzymatic activity is high, thus the system is not kinetically limited, (2) the limitation of mass transfer from the organic phase to the aqueous phase occurs in the aqueous phase, thus $Co \approx Coi$, (3) the concentration in the aqueous phase has a linear relationship with the organic phase concentration, (4) in the aqueous phase the concentration of DBT $Cw \approx 0$. Assumption 2 is Figure 2-1based on the presumption of the small diameter of the organic droplets. Assumption 3 assisted in calculating the DBT partition coefficient (K_P). Assumption 4 is based on observation that the system showed high enzymatic activity.

The model based on these assumptions was constructed by utilizing equations (4-1) and equation (4-2). Equation (4-1) which described the flux equation between the two phases, would become equation (5-1), which considers the mass transfer limitation being in the aqueous phase:

$$Flux = \mathrm{k}\,(\frac{Co}{k_p} - \mathrm{Cw}) \approx \mathrm{k}\,(\frac{Co}{k_p}) \qquad \textbf{(5-1)}$$

with, $\mathrm{Cwi} = \frac{Co}{k_p}$

By performing a mole balance for a batch system, as shown in equation (5-2), the flux of DBT into the aqueous phase is equal to the overall rate of reaction, equation (5-3).

$$-Consumption = V\frac{dCo}{dt} \qquad \textbf{(5-2)}$$

$$Consumption = r = -V\frac{dCo}{dt} = A.Flux \qquad \textbf{(5-3)}$$

$$-V\frac{dCo}{dt} = A.Flux \qquad \textbf{(5-4)}$$

Substituting equation (5-1) in (5-4) yields equation (5-5), which is the rate of consumption of DBT:

$$\frac{dCo}{dt} = \frac{-k.A}{k_p V}.Co \qquad \textbf{(5-5)}$$

Integrating equation (5-5) and setting the boundary conditions to Co = Co (0) at t = 0, and Co = Co (t), at t = t, results to equation (5-6),

$$\mathrm{Co\ (t) = Co\ (0)}\ e^{-\left[\frac{k.A}{VK_p}t\right]} \qquad \textbf{(5-6)}$$

Co (t) is the remaining DBT in the hexadecane (µmole/mL) at time t. Co (0) is the initial concentration of DBT in the hexadecane (µmole/mL). V is the hexadecane volume (mL).

The only unknown is the product (k.A), the volumetric mass transfer coefficient, which can be found from the experimental data. From the available results on DBT conversion shown in Figure 4-14, Figure 4-23, and Figure 4-25, the remaining (residual) amount of DBT was calculated. The residual DBT from the different experiments was plotted as $\ln(\frac{Co(0)}{Co(t)})$ versus reaction time. The results are shown in Figure 5-1 and Figure 5-2, for initial substrate concentrations of 1 mmole/L and 2.5 mmole/L, respectively.

Figure 5-1: Residual DBT with respect to reaction time. The initial concentration was 1 mmole/L

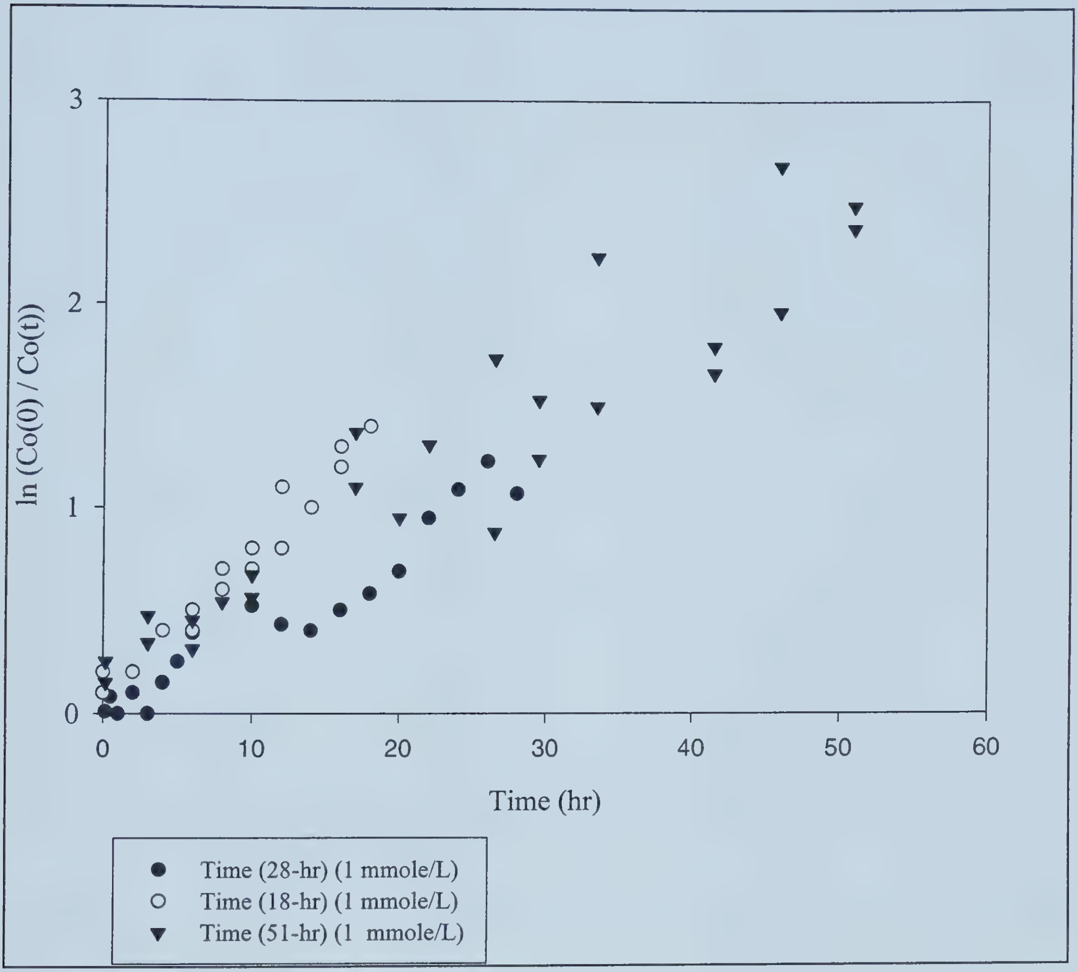
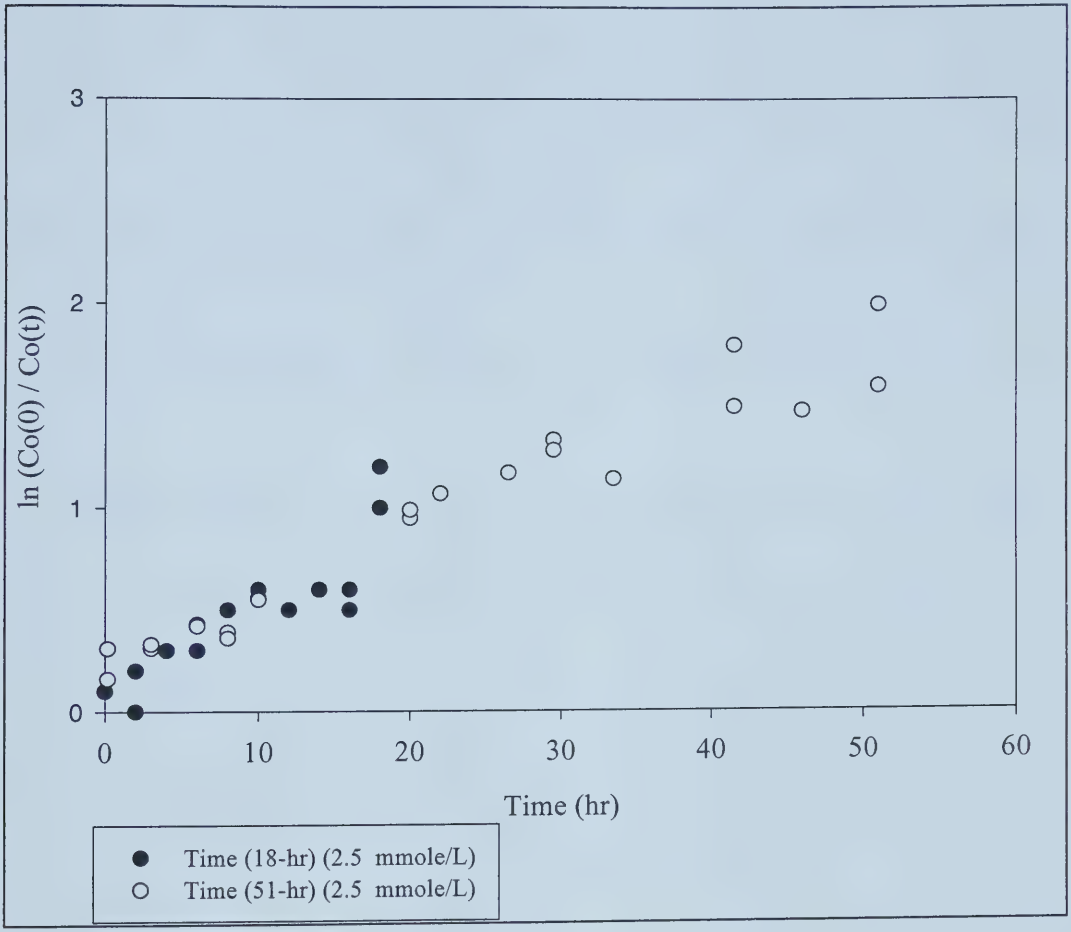


Figure 5-2: Residual DBT with respect to reaction time. The initial concentration was 2.5 mmole/L



The linear regression fitting these data had a positive slope, which indicated a first order reaction rate equation, and the parameter (k.A) was calculated. The values of (k.A) are listed in Table 5-1 for each set of experiment. Assuming the contact area A constant, the mass transfer coefficient k becomes independent of the initial concentration of DBT in hexadecane. The data in Table 5-1 were significantly close in value within the 95% confidence interval on the slopes from Figure 5-1 and Figure 5-2, except for one value. Therefore an average value of k.A = 2367 ($m^3.s^{-1}$) would be used for any further analysis.

Table 5-1: Calculated values of k.A. from the experimental data (error bounds show 95% confidence interval)

Experiment duration (hr)	Initial concentration (M)	k.A ($m^3.s^{-1}$)	Error bound on k.A
51	0.1	2574	± 394
28	0.1	2430	± 364
18	0.1	4086	± 384
51	0.25	1890	± 211
18	0.25	2574	± 682

The implication of a first order rate of conversion is its linear dependency on the DBT concentration in hexadecane. Several studies showed varying relations between mineralization of organic compounds and initial substrate concentration with and without the NAPL carrier (Rubin et al., 1982, Volkering et al., 1992, Efroymson & Alexander, 1991, Efroymson & Alexander, 1994, Efroymson & Alexander, 1995). Because of this dependency, a drop in concentration Co with time in consequence decreases the reaction rate, as shown in Figure 5-3 and Figure 5-4. This reduction was consistent with a reduction in the flux of DBT into the aqueous phase. The steady decrease in Coi affects

the Cwi value with time, as Cwi = $\frac{Co}{Kp}$, (Kp is constant). Thus the difference between the two interface concentrations become smaller causing slower partitioning of DBT from hexadecane to water. The lower concentration in Cwi consequently leads to a lower driving force for exchange into the aqueous phase. The rates of conversion in Figure 5-4 did not indicate an intercept at the origin because of the bias in the initial conversion measurement.

Figure 5-3: Rate of DBT conversion for the 51 hr experiment versus concentration of DBT in hexadecane. The initial DBT concentration was 1 mmole/L

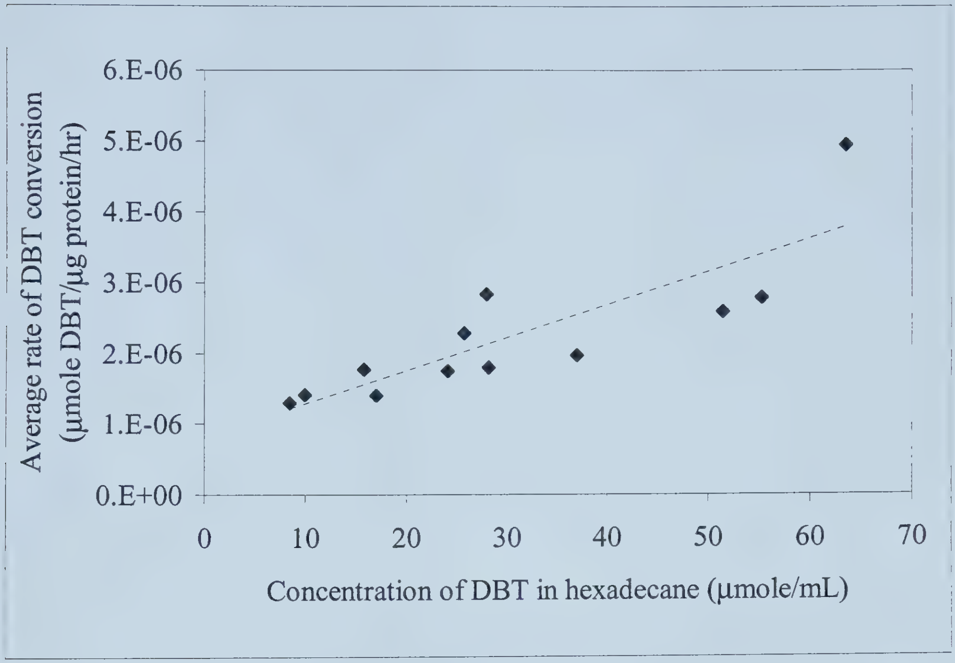
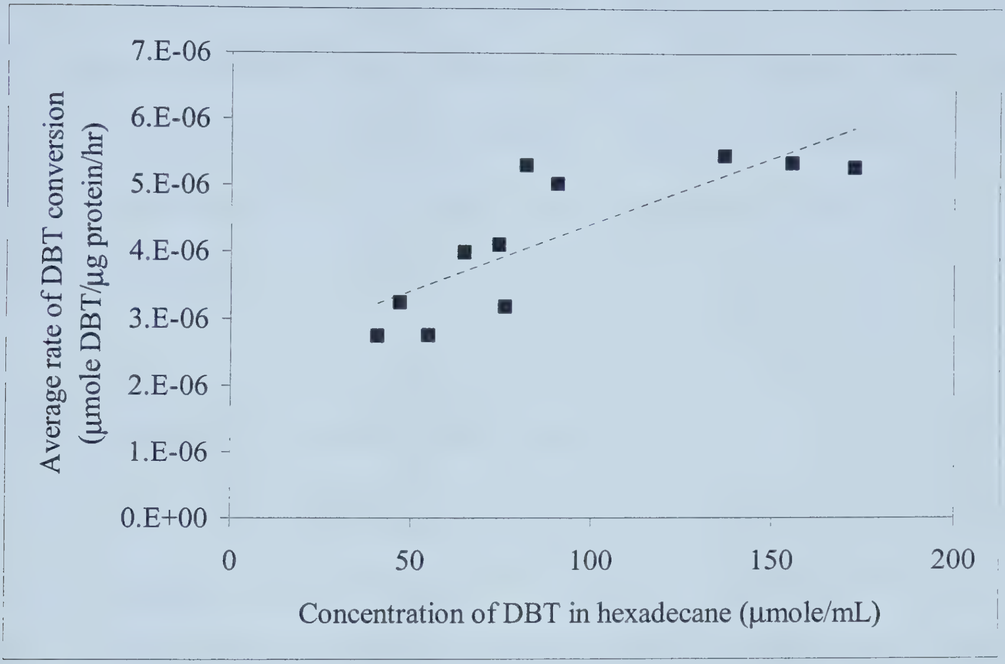


Figure 5-4: Rate of DBT conversion for the 51 hr experiment versus concentration of DBT in hexadecane. The initial DBT concentration was 2.5 mmole/L



The DBT conversion for the two concentrations (1 mmole/L and 2.5 mmole/L) was approximately 70% after 22 hr, as shown in section 4.2.3. This proportionality was also observed by Efroymson and Alexander (1995). This observation reflects a flux of DBT below the maximum rate of conversion (Simkins & Alexander, 1984). The maximum rate of conversion was not detected in this work because under the concentration range used, mass transfer rather than enzyme kinetics limited the biocatalytic system.

5.2.1 Modeling of The Initial Mean Rate

To examine the accuracy of the proposed model, the values of (k.A) in Table 5-1 were used to predict the mean of the initial rate for the first 4 hr. The predicted values were compared with the experimental values from section 4.2.5. To calculate the mean rate over the first 4 hr, the mean value theorem was used (Berkey & Blanchard, 1992). The theorem states that for a continuous function [a,b], the average value (or mean value) of the function [a,b] is the number generated from equation (5-7).

$$\bar{f} = \frac{1}{b-a}\int_a^b f(x)\,dx \qquad \textbf{(5-7)}$$

Thus from equation (5-6), the rate of disappearance of Co becomes as in equation (5-8),

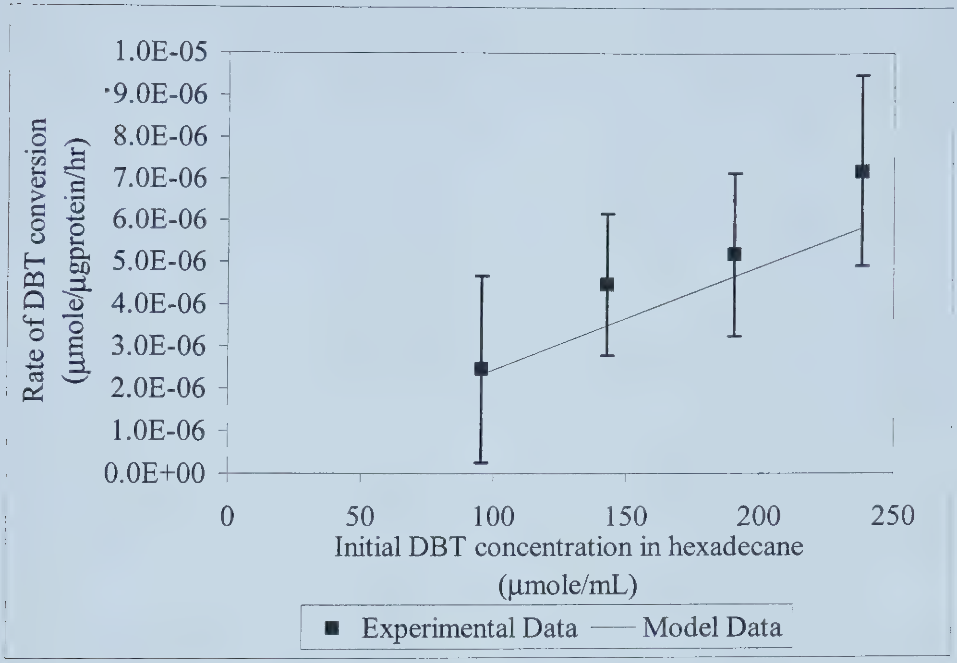
$$\frac{dCo}{dt} = -\left(\frac{k.A}{Vk_p}\right) Co(0)\, e^{-\left[\frac{k.A}{VK_p}t\right]} \qquad \textbf{(5-8)}$$

By applying equation (5-7) to (5-8), the mean rate for the first 4 hr becomes as in equation (5-9).

$$mean\ rate = \int_0^4 \frac{dCo}{dt} dt = \frac{1}{4} Co(0) \left[e^{-(\frac{kA}{Vk_p})4} - 1 \right] \tag{5-9}$$

Figure 5-5 shows the predicted values of the mean rate from equation (5-9) with k.A = 2367 ($m^3.s^{-1}$) and the experimental data for four different concentrations. The mean rate units were (μmole DBT.μg $protein^{-1}.hr^{-1}$), the protein content was ($4.5x10^5$ μg protein) and the organic volume was (0.3 mL); these data were taken from the initial rate experiment in section 4.2.5. The error bar for the experimental data were generated using the 95% confidence on the slopes of Figure 4-32. The predicted values of the mean rate were in close agreement with the experimental values.. Thus, the first order reaction rate model developed under the assumption of mass transfer at the aqueous phase was consistent with the performance of the biocatalytic system in this study.

Figure 5-5: Predicted mean rate from the model. The error bars are the 95% confidence interval



5.3 Inhibition of DBT Conversion by Metabolites

The work by Monticello et al. (1985) raised the question of whether DBT products impose an inhibiting effect on mutant 21-41. They observed that the products resulting from the oxidation of DBT were inhibitory to cell growth and further DBT oxidation. The bacterial strains they used were isolated from soil, and identified tentatively as three different *Pseudomonas* species (isolate DBT1, DBT2, and DBT3). Isolates DBT2 and DBT3 had single plasmids, which were believed to encode the ability to oxidize DBT. Monticello et al. (1985) characterized four products: 3-hydroxy-2-formylbenzothiophene (HFBT), 3-oxo-[3-hydroxy-thionaphthenyl-(2)-methylene]-dihydrothionaphthene, and the hemiacetal and *trans* forms of 4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid. The recovered products were dissolved at their original concentration in 0.1% pyruvate growth medium, a medium that supports growth. Growth on pyruvate was completely inhibited by the addition of the DBT oxidation products. In a similar experiment, the products were concentrated and dissolved at approximately their original concentrations in basal salts solution containing 0.1% DBT. GC was used to monitor the residual DBT levels. The presence of these products completely inhibited the further oxidation of DBT. The conclusion of these results depended on the mixture of the four products characterized by the study, though some of these products were spontaneously converted to HFBT. The study did not specify the extent of inhibition caused by each compound separately, and did not study the fate of HFBT with time.

Bressler and Fedorak (2001) studied the chemical stability of HFBT, and they observed that the yellow crystals of HFBT slowly turned to a reddish purple color when

stored in a sealed vial with air in the headspace. The thin layer chromatography revealed that the sealed vial had at least four distinct compounds, including HFBT. The other compounds were formed by an abiotic reaction.

When product inhibition was tested in mutant 21-41, the results showed no alteration in the activity, described as the average rate of conversion. The work by Bressler and Fedorak (2001) assisted in the design of a controlled experiment, where the metabolites were kept under the operational conditions. The metabolites were neither extracted under varying pH nor they were stored for a long time. Thus, the accumulated end products were representative of the authentic reactions products by the biocatalyst.

The different strains of Monticello et al. (1985) and the mutant in this work were expected to interact differently with the same substrate. Mutant 21-41 has a blocked degradation pathway, and does not produce HFBT as a product. Additionally, in this study the accumulation of the open ring product would be higher than the amount obtained by Monticello et al. (1985). The initial amount of DBT added was higher in Monticello et al. (1985) (1 mg/mL), and it was not converted in the presence of their oxidative products. In our work the initial amount of DBT was 0.18 mg/mL, but the percent converted in the control batches was 77% on average, and was 72% in the experimental batches with metabolites added. Despite the differences in initial concentration of DBT, we conclude that our observations contradict the observed inhibition observed by Monticello et al. (1985). The most likely explanation is that the later products of metabolism, such as HFBT, were inhibitory while the products from mutant 21-41 were not.

The fact that mass transfer was a limiting factor in this work would not affect the results on inhibition by metabolites. Mass transfer limitations would slow the conversion of DBT, but would not mask a complete loss of activity as observed by Monticello et al. (1985). The previous work also showed a complete inhibition rather than suppression, whereas this study showed no significant difference in rate of conversion. Adding a higher concentration of the stock metabolite solution might pass a threshold for detecting inhibition, but mass transfer would not be a significant factor.

5.4 Reuse and Rejuvenation of the Biocatalyst

Prior studies of the use of resting cells as biocatalysts showed that the cells required frequent addition of energy sources. For example, Naito et al. (2001) studied long term repeated biodesulfurization by immobilized *Rhodococcus erythropolis* KA2-5-1 cells. The biodesulfurization was carried out using *n*-tetradecane containing DBT as the model oil. The cells could catalyze biodesulfurization repeatedly for more than 900 hr with rejuvenation approximately every 71 hr. The amount of DBT converted in each cycle was approximately 4.35×10^{-2} (μmole DBT/ μg cell dry weight). For rejuvenation, the immobilized cells were washed, and then resuspended in medium where it was shaken for 20 hr at 30 °C. This step did not take into account the formation of newly generated cells; also the study did not provide the total amount of DBT converted. The biocatalyst in this work retained activity after the fourth 24 hr cycle, i.e. after 96 hr. Rejuvenation was needed only in one series when the biocatalyst activity dropped to lower values, as illustrated in Table 4-8. The total amount of DBT converted in this study was 3.67 (μmole/ mg cell dry weight), which is higher than in the work of Naito et al. (2001). Consequently, the sustained activity of the biocatalyst was not due to low levels of DBT conversion.

The time after which rejuvenation was needed was not fixed in this study because of the contradictory results, as shown in Table 4-8 and Table 4-13. The data of Table 4-8 were from an experiment that used a biocatalyst inoculum from a plate that was more than two weeks old and with multiple transfers of the culture. But in the later experiments

(e.g. Table 4-13), the inoculum plates were prepared 2 days before the experiment, and were prepared directly from the –70 °C glycerol stock. Consequently, the freshly prepared inoculum gave a biocatalyst that was very resistant to deactivation.

The amount of DBT converted after each 24 hr cycle was approximately the same. Thus the loss of activity could not be hidden by the mass transfer limitation, otherwise the loss of activity would build up and would show at the 3rd or 4th cycle. In addition the rate of DBT converted in each cycle was in the range of the initial rate value, as shown in Figure 5-6 and Figure 5-7, detailed description of the abbreviations was given in section 0. Figure 5-6 compares the rate of conversion of DBT when the batches were introduced to energy and reducing agent sources with the initial 4 hr rate results. The error bar for the initial rate presents the 95% confidence. Data in Figure 5-7 present the rate of conversion when the biocatalyst was repeatedly used. The results in both figures did not show evidence for activity reduction. The variations in the rate of conversion were consistent with slight differences in total protein content.

One reason for the sustained activity of the biocatalyst is that the cofactors were not consumed in the pathway used by the mutant. For the blocked pathway, as illustrated in Figure 2-11 for the DBT degradation pathways (Kodama et al., 1973), the dioxygenase enzymes would require NADH as a cofactor. The dehydrogenase enzymes in the following step would then oxidize some of the reduced NAD^+ to NADH.

Consequently, the sustained high activity of the freshly inoculated biocatalyst was consistent with the Kodama et al. (1973) pathway, which suggests a low net consumption of cofactors. Furthermore, the sustained activity indicated that the enzymes were either stable, or if inactivated they were replaced due to the presence of the inducer 2AB.

Figure 5-6: Comparison of the initial rate of conversion with the 48-72-96 hr rate of conversions. The biocatalyst was introduced to different energy and reducing agent sources. The error bar is the 95% confidence

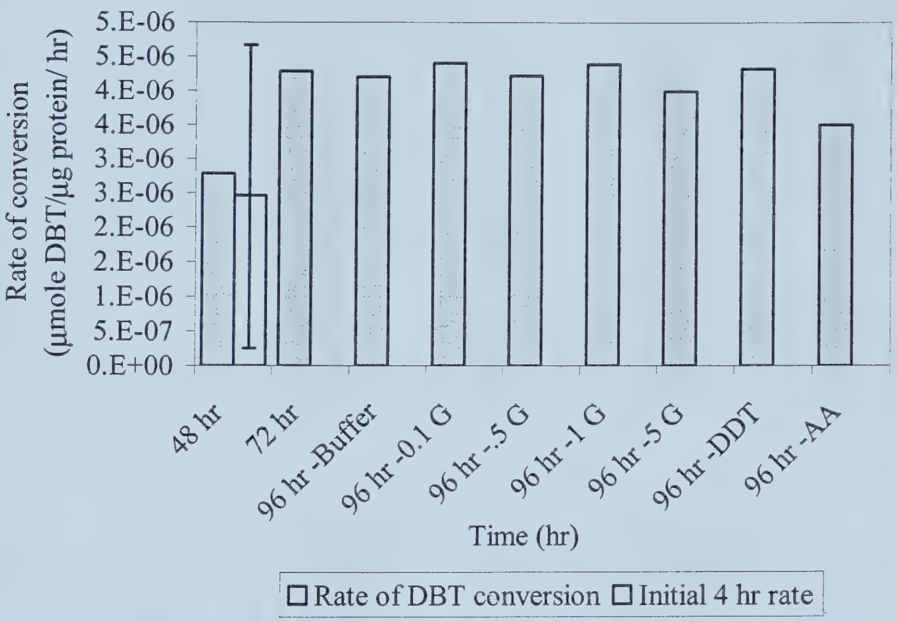
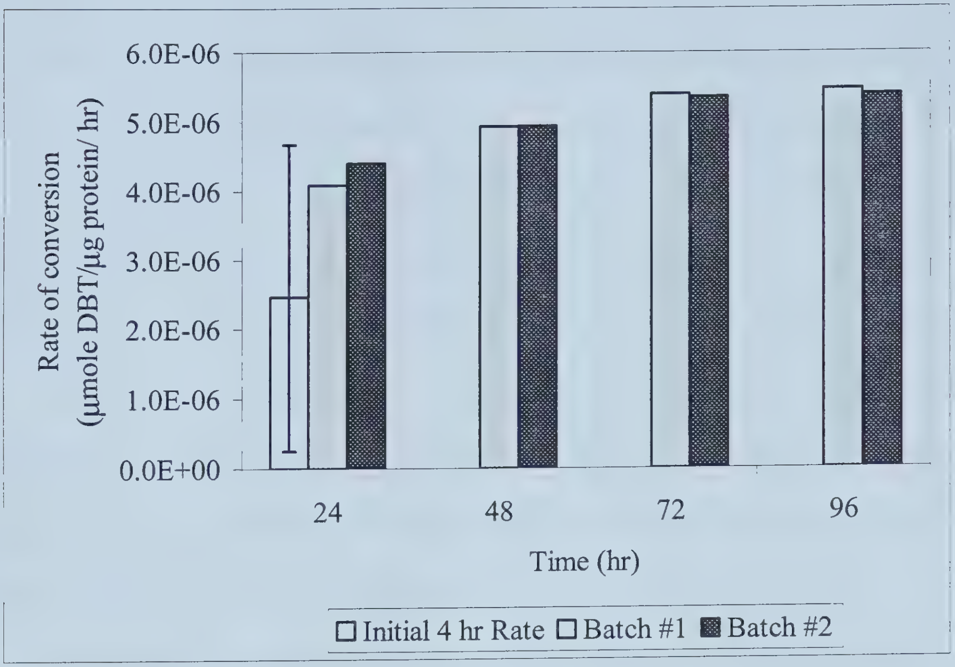


Figure 5-7: Comparison of the initial rate of conversion with the 24-48-72-96 hr rate of conversions. The error bar is the 95% confidence



6.0 CONCLUSIONS AND FUTURE WORK

The strain *Pseudomonas fluorescens* LP6a mutant #21-41 biotransformed DBT by the Kodama et al. (1973) pathway. The mutant showed a blocked degradation pathway for DBT. The end product of this blocked pathway was an open ring metabolite, which had a maximum absorbance at 475 nm. Experiments were conducted to correlate the rate of formation of the metabolite with the amount of DBT converted. The rate of formation had a varying slope with time after the initial 4 hr, because of the varying ratio of the intermediate metabolite leading to the final end product. Therefore, the correlation attempts were not successful and the moles of DBT converted were monitored by GC-FID where it showed consistent repeatability.

The ability of mutant 21-41 to convert DBT was not affected by the presence of the metabolites of DBT. The initial concentration of the metabolites varied from 10% to 90% of the biocatalyst total volume. Previous observations of inhibition were likely due to later metabolites in the degradation pathway, but since mutant 21-41 did not form these metabolites no inhibition was observed.

The biocatalyst was prepared with cells in the resting state, nevertheless, the cells sustained high activity with repeated usage. The biocatalyst was used for up to 4 cycles, and each cycle was of 24 hr. After each cycle the metabolites were removed, the biocatalyst was washed, and a fresh amount of DBT was added. The freshly prepared inoculum gave a biocatalyst with high resistance to deactivation. Furthermore, when the biocatalyst showed a very low activity, the addition of energy sources such as TSB

medium, and glucose recovered some of the activity. In degrading DBT the mutant likely had a low net consumption of cofactors, following the Kodama pathway, which is consistent with the maintenance of the high activity. Another contributing factor is that the enzymes involved were either stable, or if inactivated they were replaced due to the presence of the inducer 2AB.

The experimental data showed a linear dependency of the rate of conversion on the initial substrate concentration in the organic carrier phase. By studying the effect of varying DBT concentration in hexadecane and its concentration in the aqueous phase, the partition coefficient was calculated and was found to be 2.8×10^{-5} (mole $DBT.L^{-1}$ of hexadecane per mole $DBT.L^{-1}$ of water). By performing a mass balance on the system, it appeared that rate of conversion was equal to the flux of DBT in the aqueous phase. Thus, as the concentration of DBT decreased in hexadecane with time, the rate of conversion decreased. From the experimental data it was observed that the system also had a first order rate of disappearance. The mass transfer parameter k.A was calculated for two different concentrations (1 mmole/L and 2.5 mmole/L) with varying reaction times and had a mean value of 2367 ($m^3.s^{-1}$).

A mathematical model was developed to predict the initial rate of conversion of DBT, using the experimentally calculated values of k.A. The initial rate represented the first 4 hrs. The model was based on two assumptions: (1) the system was mass transfer limited rather than kinetically limited and (2) mass transfer limitation existed in the aqueous phase. The results from the model were compared with the experimental data, and were consistency.

From this work two main questions arise. The first is whether mutant #21-41 would sustain a high activity upon exposure to other PAC, and to diesel fuel. The second question would be the optimal mixture of nutrients needed for rejuvenation purposes.

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Appendix A - Media

Tryptic Soy Broth (Difco)

	(g/L)
Digest of Casein	17.0
Papaic Digest of Soybean Meal	3.0
Dextrose	2.5
Sodium Chloride	5.0
Dipotassium phosphate	2.5

0.1 M Phosphate Buffer pH 7

	(g/L)
KH_2PO_4	6.65
K_2HPO_4	13.35

Directions: The two compounds are mixed together until a solution of pH 7 is achieved.

Appendix B – Sample Calculations and GC Calibration

B1: Viable Count (CFU/mL)

Viable Count = Number of Colonies x f
f: Dilution factor

Example:

Number of Colonies = 83
$f = 1 \times 10^{-7}$
Viable Count = $83 \times 1 \times 10^{-7} = 8.3 \times 10^{-8}$ (CFU/mL)

B2: Dry Weight (mg/mL)

$$\text{Dry Weight} = \begin{array}{l}[(\text{Dry Weight of Sample} - \text{Weight of Empty Plate of Sample}) \\ -(\text{Dry Weight of Abiotic} - \text{Weight of Empty Plate of Abiotic})] \\ /\text{Sample Volume}\end{array}$$

Example:

Dry Weight of Sample = 1032.7 mg
Weight of Empty Plate of Sample = 987 mg
Dry Weight of Abiotic = 988.3 mg
Weight of Empty Plate of Abiotic = 978.4 mg
Sample Volume = 30 mL

$[(1032.7 - 987) - (988.3 - 978.4)]/30 = 1.193$ mg/mL

For a total volume of 200 mL of biocatalyst

Dry Weight = $1.193 \times 200 = 238.7$ mg Dry Biomass

B3: Protein Assay (μg/mL)

$$\text{Protein Concentration in Sample} = P = \frac{(A-B)-b}{m} * f$$

P: Protein Concentration in Sample (μg/mL)
A: Sample Absorbance at 570 nm

B: Blank Absorbance at 570 nm
b: Intercept of calibration curve
m: Slope of calibration curve

Example:

$m = 0.001$
$b = 0.057$
$A = 1.107$
$B = 0.002$
$f = 10$

$$P = \frac{(1.107 - 0.002) - 0.057}{0.001} * 10 = 1.05 \text{ x } 10^4 \quad (\mu g/mL)$$

For a total volume of 200 mL of biocatalyst

Total Protein Content = $1.05 \text{ x } 10^4 \text{ x } 200 = 2.09 \text{ x } 10^6$ (μg)

B4: Concentration of Chemicals in a 200mL Biocatalyst

B4-1: Preparing Salicylic Acid:

0.5M Stock Solution in 6ml DMF

$$\frac{0.5\,\text{mole}}{1000\,\text{ml}} * \frac{138.13\,\text{g}}{\text{mole}} * 6\,\text{ml} = 0.414\,\text{g}$$

0.2 mL was used for each 200 mL media of biocatalyst

$$0.2\,\text{ml} * \frac{0.414\,\text{g}}{6\,\text{ml}} = 0.0138\,\text{g}$$

Then the final concentration was

$$\frac{0.0138\,\text{g}}{200\,\text{mL}} * \frac{\text{mole}}{138.13\,\text{g}} * \frac{1000\,\text{mL}}{\text{L}} * \frac{1000\,\text{mmole}}{\text{mole}} = 0.5\,\text{mM}$$

B4-2: Preparing 2-amino Benzoic Acid:

0.5 M Stock Solution in 10 mL 95% ETOH

$$\frac{0.5\,\text{mole}}{1000\,\text{mL}} * \frac{137.14\,\text{g}}{\text{mole}} * 10\,\text{mL} = 0.686\,\text{g}$$

0.2 mL was used for each 200 mL buffer of biocatalyst

$$0.2\,\text{mL} * \frac{0.686\,\text{g}}{10\,\text{mL}} = 0.0137\,\text{g}$$

Then the final concentration was

$$\frac{0.0137\,\text{g}}{200\,\text{mL}} * \frac{\text{mole}}{137.14\,\text{g}} * \frac{1000\,\text{mL}}{\text{L}} * \frac{1000\,\text{mmole}}{\text{mole}} = 0.5\,\text{mM}$$

B4-3: Preparing DBT Stock Solutions:

0.1 M solution in different volumes of C_{16}

DBT Molarity	Solvent Added (ml)	Mass Required (g)
0.1	5	0.0921
↓	↓	↓
0.1	25	0.4607

$$0.1\,\text{M} = \frac{0.1\,\text{mole}}{1000\,\text{ml}} * \frac{184.27\,\text{g}}{\text{mole}} * 15\,\text{mL} = 0.2764\,\text{g}$$

$$0.25M = \frac{0.1 mole}{1000 ml} * \frac{184.27 g}{mole} * 15 ml = 0.6909 g$$

2 mL was used for each 200 mL buffer of biocatalyst

$$2\,\text{mL} * \frac{0.2764\,\text{g}}{15\,\text{mL}} = 0.0368\,\text{g} = 36.654\,\text{mg}$$

Then the final concentration in the total volume (aqueous + C_{16}) was

$$\frac{0.1\,\text{mole}}{\text{L}} * \frac{2\,\text{mL}}{200\,\text{mL}} = \frac{1\,\text{mmole}}{\text{L}}$$

Note-1: After 24 hr or so, the unconverted (residual) DBT in C_{16} was extracted by pentane and collected in 4 mL vials “stock for GC”.

Note-2: A sample from the (0.1 M) stock solution "original" was used also for G.C. analysis, in order to calculate the internal standard reference point (ISRF).

B4-4: Preparing G.C. Vials:

B4-4-I Internal Standards (IS)

- *Preparation*
  - 80 mg Biphenyl (Biph.)
  - 80 mg Benzothiophene (Benz.)
  - 4 mL C_6
- *Concentrations*

$$\frac{80\,\text{mg Biph.}}{4\,\text{mL } C_6} = \frac{20\,\text{mg Biph.}}{\text{mL}}$$

$$\frac{80\,\text{mg Benzo.}}{4\,\text{mL } C_6} = \frac{20\,\text{mg Benzo.}}{\text{mL}}$$

Note: The 4 mL C_6 has both substrates mixed.

B4-4-II Each G.C. Vial Contains

Example:

- 900μl DCM
- 100μl Sample of DBT "from stock or residual sample" DBT/C_{16}
- 50μl IS

Note: The sample mixture is diluted 10 times

B4-4-III Concentration of each Compound

$$[\text{IS}] = \frac{\frac{20\,\text{mg}}{\text{mL}} * 50\mu\text{L}}{1050\mu\text{L}} = 0.952\frac{\text{mg}}{\text{mL}}$$

$$[DBT] = \frac{\frac{276.4\,mg}{15\,mL} * 100\,\mu L}{1050\,\mu L} = 1.754\frac{mg}{mL}$$

B4-4-IV GC Data

There were 3 peak areas at retention times (Rt.):

Rt. ≈ 9.4 min Correspond to Biph.

Rt. ≈ 6.99 min Correspond to BT.

Rt. ≈ 13.9 min Correspond to DBT

The other peaks corresponded to C_{16} and DCM

B4-4-V Analyzing the GC Data

1- *Internal Standard Reference Point "ISRF"*

$$ISRF = \frac{[DBT]}{[IS]} * \frac{1}{n}\sum \frac{\text{Area of IS}}{\text{Area of DBT}}$$

[DBT] = Concentration of DBT in GC vial (from the original stock)
[IS] = Concentration on internal standard (either Biph. Or BT.)
n: The number of GC vial prepared from the same main sample (e.g. 2 GC vials from the 0.1 M original sample).
$\sum$: The summation of the (n) G.C. vials from the same main sample.

2- *Amount of the Residual DBT in GC vials*

$$\text{Amount of Residual DBT} = [IS] * ISRF * \frac{1}{n}\sum \frac{\text{Area of Compound}}{\text{Area of IS}}$$

Example:

1- Calculating the ISRF
2- Calculation of residual DBT in G.C. vials and the biocatalyst
3- Calculating DBT conversion and rate of conversion

Table B-1 Resulted GC areas for the stock (0.1 M) DBT

Compound	Vial 1	Vial 2
Biph.	11551.3	9716.74
DBT	15831	16984.5
DBT/Biph.	1.370	1.748
Biph/DBT	0.73	0.572

Note: 2 vials were prepared to present the same sample

$$\text{ISRF} = \frac{1.755}{0.952} * \frac{1}{2}(0.73 + 0.572) = 1.199$$

Table B-2 Resulted GC areas for samples of residual DBT

Compound	Vial 1	Vial 2
Biph.	9505.21	9806.93
DBT	11003.6	10900.9
DBT/Biph.	1.158	1.112
Biph/DBT	0.864	0.90

$$\text{Amount of Resiual} = 0.952 * 1.199 * 0.5 * [1.158 + 1.112] = 1.295 \frac{\text{mg}}{\text{ml}}$$

$$\text{Undiluted Residual Sample of DBT} = 12.95 \frac{mg}{ml}$$

Residual Mass of DBT = Undiluted Residual Sample of DBT * Volume Added to a 200 mL biocatalyst

Note: Since 98.9% of the organic phase is recovered then, instead of using 2 mL in the further calculations, only a 0.989 * 2 mL = 1.978mL is going to be used.

$$\text{Residual Mass of DBT} = 12.96 \ \frac{\text{mg}}{\text{mL}} * 1.978 \text{ mL} = 25.635 \text{ mg}$$

Converted Mass of DBT = Added Mass of DBT * Residual Mass of DBT

Converted Mass of DBT = 1.755 * 2 mL * 10 - 25.635 = 9.465 mg

% Conversion (no extraction loss) =

$$\frac{35.1-(12.96*1.978)+(12.96*2-1.978)}{35.1}*100 = 26.2\%\ \text{wt/wt}$$

% Conversion (estimated extraction loss) =

$$\frac{35.1-(12.96*1.978)}{35.1}*100 = 26.9\%\ \text{wt/wt}$$

B4-4-VI Calculating DBT Conversion and Rate of Conversion

For a Total Protein Content = $10^6\ \mu g$

The Standard Unit for Conversion of DBT is $\frac{\mu\text{moleDBT}}{\mu\text{g protein.}}$

The Standard Unit for Rate of Conversion is $\frac{\mu\text{moleDBT}}{\mu\text{g protein. hr}}$

Converted Mole of DBT = Converted Mass of DBT / Molecular weight of DBT

$$= (9.465 / 184.27) * 1000 = 51.36\ \mu\text{mole}$$

Conversion of DBT = Converted Mole of DBT / Total Protein Content

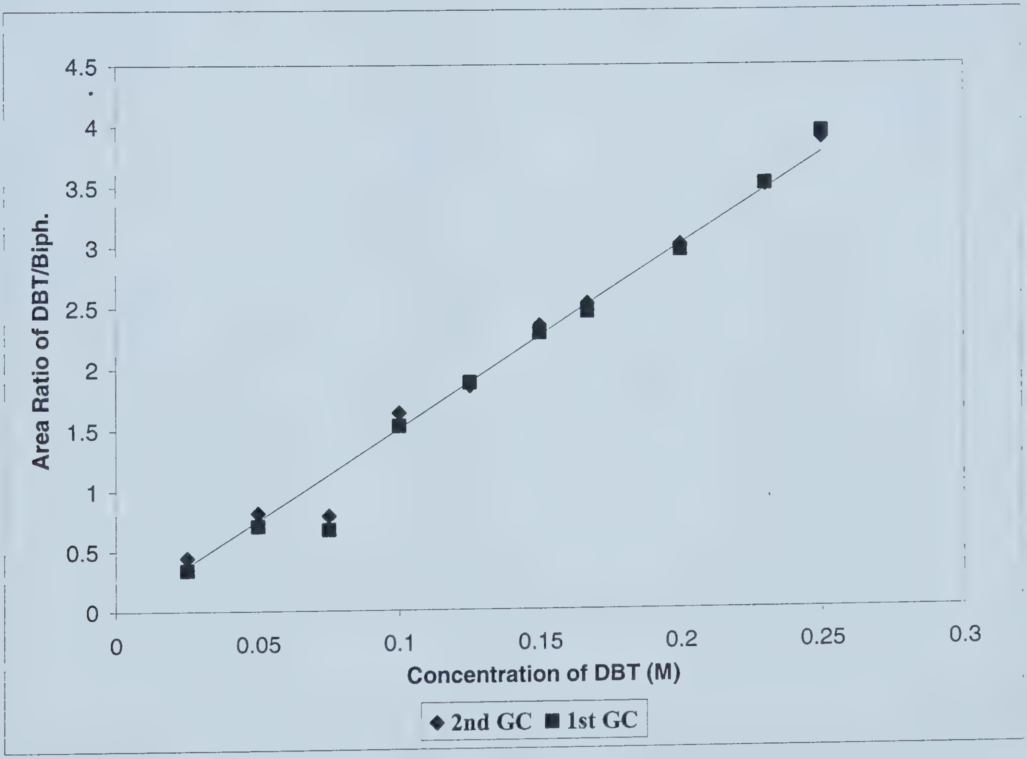
$$= 51.36 / 10^6 = 5.136 \times 10^{-5}\ \frac{\mu\text{moleDBT}}{\mu\text{g protein.}}$$

Rate of Conversion = Conversion of DBT / Reaction Time

$$= 5.136 \times 10^{-5} / (24 - 0.166) = 2.14 \times 10^{-6}\ \frac{\mu\text{moleDBT}}{\mu\text{g protein. hr}}$$

B5: Calibration Curve of the GC

The stock solution of DBT had concentrations varying from 0.1 M to 0.25 M. Therefore the GC was tested for its accuracy within this range, using a suitable temperature program. Two GC machines were used for the analysis. For both machines the standard curve was linear within the experimental range of concentrations used.



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